# Observations on the abomasal proteome during *Teladorsagia circumcincta* infection in sheep

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# Declaration

I certify that the work in this thesis has not been previously submitted for a degree, nor has it been submitted as part of requirements for a degree or other recognised award. I also certify that the thesis was written by me. Any help that I have received in the course of my research has been acknowledged. Information sources and literature references are acknowledged and indicated in the thesis.

---------- G M Goldfinch

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# Abstract

*Teladorsagia circumcincta* is a major financial burden on the UK sheep farming industry. Disease control is becoming increasingly difficult due to the rapid emergence of anthelmintic resistance. This has prompted the search for alternative, sustainable control measures, including vaccination. Vaccine design would be aided by a thorough knowledge of the mechanisms involved in immunity to *T.circumcincta*. Most research has focussed on humoral and cellular responses to infection with this nematode. This thesis focuses on the impact of infection with regards to the proteins found locally within the abomasum.

Using a well established infection model, proteomic analysis of lymph draining the abomasum was carried out by means of 2-dimensional electrophoresis (2-DE). The identity of many of the proteins in gastric lymph was revealed by means of MALDI-TOF analysis. The relative quantities of the lymph proteins were monitored over time using gel analysis software in both primary infection and immune challenged infection models. This study revealed a number of proteins of interest, including the acute phase proteins serum amyloid A, alpha-1 acid glycoprotein and haptoglobin, as well as the actin depolymerising protein, gelsolin. The effect of infection and immunity to *T.circumcincta* on these proteins was investigated further by means of biochemical assays, western blotting and real-time PCR. The impact of infection on the permeability of the abomasal mucosa will affect the resultant gastric lymph proteome. This "leak lesion" phenomenon is well documented in *T.circumcincta* infection but the underlying cause is unknown. Tight junction proteins in the abomasum were studied, using immunofluorescence techniques, in an attempt to define the role of these proteins in this important immunological/pathological event.

The aim of this thesis is to contribute to the knowledge of innate immune responses and local pathology occurring within the abomasum during *T.circumcincta* infection.

# 1 Chapter 1: General Introduction

The parasitic nematode, *Teladorsagia circumcincta*, is of major importance to the UK sheep farming industry due to its involvement in the clinical syndrome known as parasitic gastroenteritis. It is the main nematode species responsible for this disease in temperate regions and is a financial burden on the industry, through both a direct reduction in production efficiencies, and also through the costs incurred in the chemical treatment and prevention of the disease.

Adequate control of this disease has been achieved in the past through the strategic use of anthelmintics throughout the year. However in recent years, strains of this parasite, resistant to the available anthelmintic drugs, are becoming increasingly common (Bartley *et al.* 2004; Wrigley *et al.* 2006) and in light of such resistance the parasite is being given the opportunity to flourish on those farms affected. The emergence of new anthelmintics is limited with only one novel class, the amino-acetonitrile derivatives (AADs) (Kaminsky *et al.* 2008a), approaching availability in the UK. Intensive sheep farming globally is facing a crisis and, as such, alternative strategies for controlling parasitic nematodes are being sought and *T. circumcincta* is not an exception. One such alternative would be vaccination of lambs, preventing the development of clinical disease upon exposure to the worms. In order to aid vaccine design, it is important that we better understand the immune mechanisms and parasite antigens involved in protective immunity to, and in expulsion of, this parasite from the abomasum. This thesis aims to contribute to our knowledge of the immune mechanisms taking place within the local tissues of the abomasum in the face of infection with *T.circumcincta*, with particular interest in protein changes detectable in the lymph draining the affected organ.

## *1.1 Teladorsagia circumcincta: background and lifecycle*

*Teladorsagia circumcincta* (formerly known as *Ostertagia circumcincta*), is a helminth of veterinary importance. It belongs to the class Nematoda and is a

member of the superfamily, Trichostronglyloidea. Other important veterinary nematodes from this superfamily include *Ostertagia ostertagi* (parasitic gastroenteritis – Bovine ostertagiosis – in cattle), *Haemonchus contortus* (anaemia and production loss in sheep –Haemonchosis), *Nematodirus battus* (the cause of a specific gastroenteritis in sheep), and *Dictyocaulus viviparus* (cattle lungworm).

Like the other worms in the Trichostrogyloidea superfamily, *T.circumcincta* has a direct life-cycle and does not rely on any intermediate host for transmission of larval stages. Figure 1.1 shows a simple diagrammatic representation of the life-cycle of this parasite.

The predilection site within the ovine host is the abomasum, and it is here that the adult worms mate, with the production of eggs by the females. These eggs are passed out in the faeces on to pasture. Within the faecal pat, the lifecycle continues with the egg developing through two larval stages to become the infective third larval stage, or L3. The L3 then migrate out of the faecal pat onto the pasture, and in this way increase their chance of consumption by another potential host. The process of development to L3 and migration onto pasture occurs at varying rates depending on climatic factors, especially extremes of temperature or moisture (Urquhart *et al.* 1996).

Once ingested by a suitable host, the L3 larvae exsheath within the rumen before settling at their predilection site, the gastric pits within the abomasum. Here a further two parasitic moults take place, and approximately 18 days after infection with L3 (although this does vary – see later), the fifth stage adults emerge onto the surface of the abomasum where sexual maturation, mating and egg production occurs.

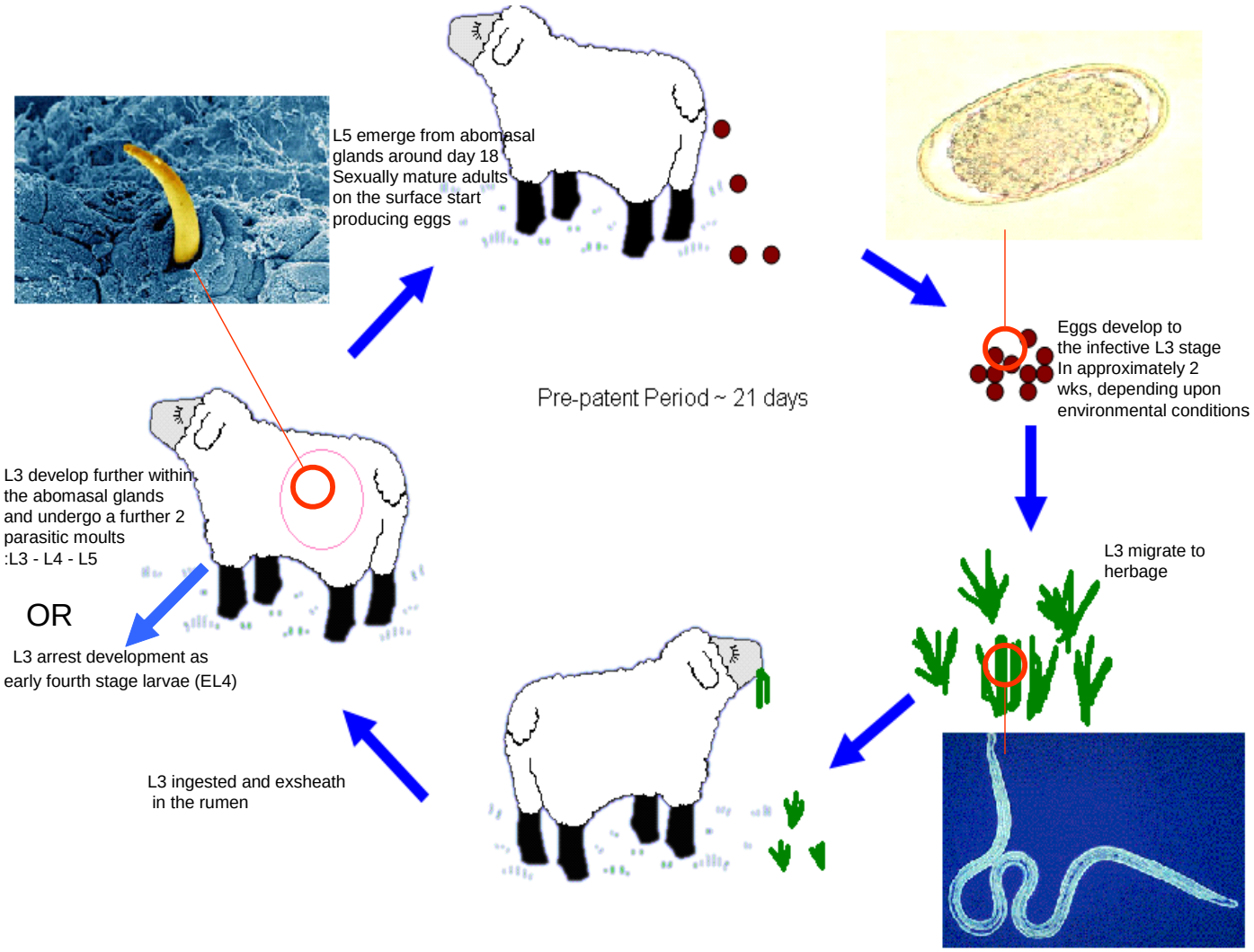



**Figure 1.1 Simple diagrammatic representation of the life-cycle of the *T.circumcincta* parasite**

**Picture credits: L3: Sinclair Stammers, Imperial College, London**

**: Trichostrongyle Egg: Irish Agricultural and Food Development Agency**

**: L5 emerging from abomasal gland: Moredun Research Institute**

## *1.2 Epidemiology*

Residual pasture contamination, resulting from the survival of overwintered larvae from the previous grazing season, contributes to disease in lambs the following year. This is dependent on type of pasture, climatic conditions and stocking rates (Crofton 1957). Young animals predominantly are infected as immunity usually only develops at about 6 months of age (Smith *et al.* 1985).

In Scotland, a number of studies have indicated that each species of parasite follows a definite seasonal pattern. *Nematodirus battus* is one of the first species to establish an infection each year (Connan 1968; Crofton 1954; Crofton 1957). *T. circumcincta* is the most common abomasal nematode in the spring and early summer (Connan 1968; Crofton 1954; Crofton 1957) accounting for up to 90% of the total worm burdens (Reid & Armour 1975b) while *H. contortus* occurs sporadically in small numbers during this time (Connan 1968; Crofton 1954; Crofton 1957). *T. vitrinus* is the most prevalent nematode found in the small intestine with the highest level of infection occurring during the late summer months although the numbers were consistently low (Connan 1968; Crofton 1954; Crofton 1957). It is important to note that there is always a number of species present at any one time. This seasonality is also subject to the influences of climate change (van Dijk J. *et al.* 2008; Wall & Morgan 2009).

Studies by Crofton (Crofton 1954; Crofton 1957), Connan (Connan 1968) and Reid and Armour (Reid & Armour 1975b; Reid & Armour 1975a) also reported evidence of the 'spring rise' in faecal egg counts in ewes, a well documented event in which the faecal egg count of ewes rises dramatically in the spring. This phenomenon has been attributed to the maturation of previously inhibited larval stages, the development of recently ingested larvae and changes in environmental conditions enabling eggs and larvae which over-wintered on the pasture to resume their development (Crofton 1954; Crofton 1957). It is also thought that the ewe's immunological response is temporarily relaxed, due to the stresses of the winter

followed by lambing, lactation and subsequent weaning (Reid & Armour 1975b; Reid & Armour 1975a).

This is true for any gastrointestinal nematode infection for which the larvae can successfully overwinter. In the UK, the principal genera for which this epidemiological facet applies are *Teladorsagia* and *Nematodirus* (Boag & Thomas 1977). It is thought that faecal pellets act as reservoirs for infective larvae, and it is these which allow survival of the larvae over the winter period and hence ensure the infectivity of the pasture for a prolonged time (Gibson & Everett 1972). In the case of *T.circumcincta*, it has been suggested that the consumption of these larvae by susceptible lambs results in a peak of infective larvae being available on pasture in August (Gibson & Everett 1973). The actual timing of this peak is open to influence from climatic conditions, in particular temperature and moisture, both of which influence the rate of development of the free-living stages of the parasite.

Gibson et al (Gibson & Everett 1973) demonstrated that a temperature of 10$^{o}$C and adequate moisture were necessary for the development of free-living stages, and that low spring temperatures would subsequently delay development and hatching of the nematode eggs (Gibson & Everett 1976), and hence the period when the infective larvae were available on the pasture. The time taken from egg to infective L3 on pasture is dependent on environmental conditions and this has again been demonstrated, with a UK study designating 14-84 days to this process (Gibson & Everett 1972), and a comparable Australian study concluding that this free living life-cycle stage would be completed in 4-27 days (Callinan 1978). L3 survival on pasture is subsequently limited by hot dry summers.

The main source of infection to young lambs (the group most affected by parasitic gastroenteritis) is pasture contamination from ewes suffering from a waning of immunity during pregnancy (O'Sullivan & Donald 1973; O'Sullivan & Donald 1970). This leads to recrudescence of arrested larvae, and also allows the development of overwintered larvae ingested by the ewes, with the production of viable nematode eggs (Reid & Armour 1975b). Reid et al., (Reid & Armour 1975b),

found that nematode burden and faecal egg count increased in late pregnancy, but reached a maximum level during lactation. Although the peri-parturient rise (PPR) (or "spring rise") is predominantly a feature of the pregnant/lactating ewe, it also occurs to a minor extent in barren ewes and male sheep (Brunsdon 1964; Crofton 1958). The infective larvae made available because of this peri-parturient rise lead to two peaks in pasture infective larvae – June and August (Gibson & Everett 1973). The June peak is thought to be a direct consequence of the "spring rise", while the August peak is a result of lambs ingesting infective L3 in June.

## *1.3 Pathology and Clinical Signs*

### 1.3.1 Production loss

*Teladorsagia circumcincta* causes economic loss largely through reduced production. It is principally a disease of lambs in their first grazing season and the loss of production is demonstrated by reduced skeletal growth, slower deposition of bone mineral (calcium and phosphorus), reduced weight gain and hence longer time to slaughter weight (Sykes *et al.* 1977). Sykes et al. (1977) also noted that infection with the nematode is accompanied by reduced food intake and hence proposed that the associated production loss is due to induced energy or protein deficiency. Of economic importance is the fact that, even in the absence of overt clinical signs, constant exposure to relatively low larval challenges (1000 larvae/day), can have a negative effect on production performance (Coop *et al.* 1977).

### 1.3.2 Biochemical changes

Larval challenge is associated with raised serum pepsinogen and also mild hypoalbuminaemia in animals receiving a moderate challenge (4,000 L3/day) (Coop *et al.* 1977; Simpson 2000). In animals undergoing a single challenge, it was found that serum pepsinogen elevation and appetite suppression coincided with emergence of the developing larvae from the glands (peak around day 16 post infection)

(Holmes & Maclean 1971). This result also supports the hypothesis put forward by Coop et al. (Coop *et al.* 1977), that it is the L4 larvae, and not the adults, which are the most pathogenic stage of *T.circumcincta* infection - a point further validated by the poor correlation between FEC and the pathogenicity of infection. When animals were given a continuous challenge (which better mimics the field situation) the serum pepsinogen elevation and reduced appetite were present throughout, suggesting continuous damage and development of the larvae.

Raised serum pepsinogen is thought to be due to the development of "leak lesions" in the abomasal mucosa, whereby the abomasal mucosal barrier becomes less competent due to damage inflicted by the nematodes (McLeay *et al.* 1973). This "leak" phenomenon may be advantageous to the sheep, allowing the passage of substantial amounts of antibody which can then gain direct access to the parasite. The actual histopathology involved remains unclear, but may involve rearrangement or loss of crucial tight junction proteins, which normally control the movement of substances in the paracellular pathway.

### 1.3.3 Histological changes

Studies looking at histological changes which occur in the abomasum with infection have found a generalised hypertrophy of the abomasal fundic mucosa (Coop *et al.* 1977) as well as a reduction in parietal cell numbers (the degree of which appeared to correlate with the size of challenge) (Coop *et al.* 1977; Scott *et al.* 2000). Lesions intimately associated with the invasion of glands have been reported, namely gland expansion and loss of differentiation of cells within the affected gland and in neighbouring glands (Coop *et al.* 1977). This loss of differentiation affects physiologically important cell types, including the acid producing parietal cells and the pepsinogen secreting zymogenic cells (McKellar 1993). Infection is also associated with the accumulation of inflammatory cells, such as eosinophils and neutrophils (Coop *et al.* 1977; Scott *et al.* 2000)

Anderson et al. (Anderson *et al.* 1976) suggested the likelihood of increased circulating gastrin, probably as a response to reduced acid output from the parietal cells in the affected regions of mucosa. The changes in parietal cell function in the infected abomasum are further demonstrated by the increase in abomasal pH observed during a single challenge with 100,000 larvae (Thomas & Waller 1975). In this experiment, pH increased from normal (pH 3-3.5) to pH 6.3 by day 8 post challenge. The elevated pH was then maintained for 35 days following a single challenge. The rise in pH favours the survival of anaerobic bacteria and in turn these may compromise host nutrition (Simpson 2000) through protein degradation. This change in abomasal pH also has the potential to significantly alter the enzymatic digestive physiology of the affected sheep and reduce food conversion efficiency, further augmenting production loss caused by this parasite.

## *1.4 Manifestations of Immunity*

It is well known that sheep continuously exposed to *T.circumcincta* in a natural pasture setting do develop immunity to the parasite. This is shown on a clinical level by the fact that parasitic gastroenteritis is largely a problem of animals in their first grazing season, and is seen rarely in adult animals. The actual manifestations of immunity would appear to be numerous. As well as a loss of established worms and resistance to the establishment of new populations, immunity is also demonstrated by inhibition of worm development, stunting and reduced fecundity (ovulation) of females. Hence, immunity is shown by a reduction in faecal egg counts (FEC), a greater proportion of arrested L4 larvae, and lower worm burdens (Seaton *et al.* 1989).

Although recognised as one consequence of immunity, a number of other factors are thought to be important in causing L4 larvae to arrest in development. These include the environmental conditions the L3 were exposed to on pasture, the worm population already existing within the abomasum, as well as the immune status of the host (Armour *et al.* 1969a; Armour *et al.* 1969b). Gibson et al. (Gibson & Everett

1973) found that the time of commencement of larval arrest was the same in both lambs and ewes, adding weight to the hypothesis that arrest is not solely a function of the immune status of the host.

### 1.4.1 Peri-Parturient Rise

The peri-parturient rise in FEC is essential in the epidemiology of this parasite and is an indication of immunity in the pregnant ewe temporarily waning. The mechanism behind the re-emergence of inhibited larvae during the PPR is not clear, but it is thought that nutritional demands of pregnancy and lactation may play a role in disrupting the existing protective immunity (Houdijk *et al.* 2001a; Houdijk *et al.* 2003). Indeed it has been shown that protein supplementation during late pregnancy and lactation can actually reduce FEC and worm burdens (Houdijk *et al*. 2000).

### 1.4.2 Exposure and immunity

The ability of the lamb to resist infection occurs after 10 weeks of continuous infection, typical of the natural grazing setting in the UK during the summer months (Gibson & Parfitt 1976). The development of immunity appeared to be unaffected in these studies by various anthelmintic regimes, designed to mimic the variety of protocols which may be applied in the field. Interestingly however, even regular anthelmintic use did not completely diminish production loss as a result of *T.circumincta* challenge. This may be an area in which vaccination can surpass the performance of anthelmintics. On the other hand, the possibility that the development of protective immunity necessitates a sacrifice in production cannot be ignored.

A similar time scale for development of immunity was proposed by Seaton et al. (Seaton *et al.* 1989). They found that immunity manifested itself as retardation of worm growth and development between 0-4 weeks post challenge, worm arrestment at EL4 between 4-8 weeks post challenge, and then increased worm expulsion at 8-12 weeks post challenge.

### 1.4.3 Age-related Immunity

Another important characteristic of immunity to *T.circumcincta*, is the relative inability of the young lamb to mount an effective immune response against the parasite, compared to its older counterpart (Smith *et al.* 1985). Smith et al (Smith *et al.* 1985) showed that in 4.5 month old lambs, both lymphoblast and IgA responses were poorly developed compared to their 10 month old counterparts. It was shown previously that the ability of the lamb to mount an effective and protective immunity to *T.circumcincta* is well developed by 36 weeks of age (Gibson & Parfitt 1972). This age immunity phenomenon will become a very important area should vaccination against the disease become a feasible option. In particular, mucosal immunity, as measured by cellular and humoral responses in gastric lymph, is less well developed in the young host (Smith *et al.* 1985). Since, however, it has been shown that IgA is not responsible for worm expulsion in the immune individual, it is likely that another, as yet undetermined factor or factors, will be contributing to this age related effect (Halliday *et al.* 2010; Halliday *et al.* 2007; Smith *et al.* 1984).

## *1.5 Immune mechanisms*

### 1.5.1 T-helper type-2 (Th2) bias

It is now well accepted that immunity to gastrointestinal helminth infections is mediated through a Th2 pathway. Much of the knowledge regarding immune mechanisms in helminth infection has arisen from the use of mouse models, particularly immunity to *Trichinella spiralis*. Specifically, Th1 cells expressing IFN-γ, IL-12 and IL-18 have been shown to increase host susceptibility to helminth infection as measured by helminth persistence (Artis 2006a; Artis & Grencis 2008). In contrast, Th2 cells and associated cytokines (IL-4, IL-5, IL-9, IL-13, IL-25 and IL-33) have been associated with resistance to infection in mice (Artis 2006a; Artis & Grencis 2008).

From mouse models it is thought that IL-4 and IL-13 may provide the basis of this Th2 controlled immunity through effects on fluid secretion in the gut, goblet cell responses and smooth muscle contraction (Artis 2006a). Activation of the transcription factor Stat6 (signal transducer and activator transcription factor 6) by these Th2 cytokines, IL-4 and IL-13, in turn leads to goblet cell hyperplasia and resultant mucus production (Khan et al. 2001). Indeed, Stat6 knockout mice had lower numbers of goblet cells and slower worm expulsion (*T.spiralis*) from the GI tract. Expulsion of *Nippostrongylus brasiliensis* was also found to be IL-4, IL-13 and Stat6 dependent (Urban, Jr. *et al.* 1998).

Following on from the studies in murine models, the Th2 bias of the immune response to nematode infection has also been verified in sheep. Intestinal lymph cells from sheep selected for resistance to *Trichostrongylus colubriformis* have showed increased expression of IL-5, IL-13 and tissue necrosis factor alpha (TNF-α) (Pernthaner *et al*. 2005). More recently, expression of cytokines in the gastric lymph node of *T.circumcincta* infected sheep has also shown significant increases in IL-4, IL-5 and IL-13 (Craig *et al*. 2007), indicative of a Th2 response.

### 1.5.2 Mast cells

Mast cells are multi-functional tissue dwelling cells of bone-marrow origin. They contain a variety of biological mediators, stored within granules, which can be released by different stimuli, including antigen (via high affinity IgE receptors), complement proteins and other inflammatory mediators (Krishnaswamy *et al*. 2001). The actual mechanisms involved in immunity and their relative impact on the overall ability of the animal to resist nematode infection are not yet well understood. The idea that worms could be rapidly expelled within 48 hours without actually settling in their predilection site was suggested by Miller and Jackson, (Miller *et al*. 1983). It was thought that this was due to a hypersensitivity reaction involving mucosal mast cell degranulation. This view was supported by the observation that a mast cell derived protease (sheep mast cell proteinase, SMCP) was shown to be present in much higher levels in immune sheep versus parasite naïve animals (Miller & Huntley 1982). Further evidence to support a central role for mast cells in immunity to

worms lies in the findings of Knight et al (Knight *et al*. 2000) who showed the delayed expulsion of the GI nematode *Trichinella spiralis* in response to deletion of the mouse mast cell protease-1 gene, in comparison to its wild type counterpart. However, the relative importance of these mast cell proteases may differ depending on the exact nature of the nematode infection, and may not, in fact, be of major importance in the expulsion of certain worm species. This is demonstrated by the lack of effect of MMCP-1 gene deletion on the ability of the mouse to clear *Nippostrongylus brasiliensis* infection. Infection studies with *T. circumcincta* in ovine and caprine hosts showed higher MCP levels in sheep compared to goats, with sheep being more effective at excluding nematode infections (Macaldowie *et al*. 2003). In addition, negative correlations have been demonstrated between mucosal mast cells, globule leukocyte counts (end stage mast cells, usually found intracellularly (Huntley *et al.* 1984)) and FEC in the peri-parturient ewe (Houdijk *et al.* 2003).

Mast cell products may have a direct effect on the parasites, or possibly increase permeability of the gastric mucosa to macromolecules, immune effectors, from the circulation and extracellular fluid. Increased permeability may arise through the opening of apical tight-junctions, and the development of paracellular permeability as has been shown in rat jejunum during anaphylactic release of RMCP-II (Scudamore *et al*. 1995). Evidence suggests that this also occurs in immune sheep as indicated by a temporary increase in lymph and blood plasma pepsinogen following challenge (Lawton *et al.* 1996; Smith *et al.* 1984).

### 1.5.3 Eosinophils

Eosinophils have been linked to parasite rejection and are known to be part of the anti-parasite immune response (Meeusen *et al.* 2005a). In both *H.contortus* and *T.circumcincta* infection, the number of eosinophils in abomasal tissue increases significantly following challenge (Balic *et al.* 2000; Balic *et al.* 2003). In immune animals, this increase in eosinophil number occurs rapidly, being apparent only three days after challenge with *T.circumcincta* (Balic *et al.* 2002; Balic *et al.* 2003). Interestingly, the peak in eosinophil number coincides with the peak in

*T.circumcincta* larvae (Balic *et al.* 2003). Balic et al. (Balic *et al.* 2000) suggested that the eosinophils were a specific response to the larval nematode stage, with numbers decreasing when the parasites had matured to adults.

The actual function of the eosinophils may be through a direct effect on the nematode larvae themselves. This is suggested by the close spatial association observed between eosinophils and *H.contortus* larvae (Balic *et al.* 2002; Balic *et al.* 2006). *In vitro* studies involving the incubation of *H.contortus* larvae with eosinophils, in the presence of antibody against a defined L3 surface antigen (*Hc*sL3), resulted in larval immobilization and significant larval killing after 24 hours (Rainbird *et al.* 1998). Ultrastructural analysis showed degranulation of eosinophils adhering to the larvae, with possible damage to the cuticle of the worm (Rainbird *et al.* 1998). It was found that the anti-parasite effect of eosinophils was improved by the addition of complement or IL-5, as well as by repeated *in vivo* stimulation by L3 larvae (Rainbird *et al.* 1998). These data suggest that eosinophil activation and subsequent larval killing may be reliant on a suitable environment, including cytokines, a point also suggested by Balic et al. (Balic *et al.* 2006; Meeusen *et al.* 2005a). It may be that this environment is more readily present in the immune sheep.

### 1.5.4 Mucus

Mucus on the surface of gastrointestinal epithelium also has a role in the expulsion of nematode parasites. As well as the physical barrier that mucus viscosity provides, a property that is altered by the inclusion of molecules such as IgA and albumin (Miller 1987)**,** mucus also contains important molecules, which may directly interact with the parasite. Gastric mucus in man is mainly composed of the mucins MUC5AC and MUC 6 (Nordman *et al.* 2002), glycoprotein products of goblet cells which contribute to the barrier function of mucus. Mucus also contains other molecules related to the underlying mucosa, including antibodies, enzymes, plasma proteins and secretions from underlying glands such as pepsinogen and gastrin (Miller 1987)**.** The antibody class most abundant in mucus is IgA.

### 1.5.5 Immunoglobulins : IgA, IgG and IgE

The exact importance of antibodies in the process of worm expulsion and immunity to *T. circumcincta* is not clearly understood. Mucus from sheep infected with the intestinal nematode *Trichostrongylus colubriformis* has been shown to contain IgA antibody specific to an L3 surface antigen. This antibody causes *in vitro* clumping of larvae and hence may be expected to prevent larvae from establishing in their predilection site *in vivo* (Harrison *et al.* 2003b; Harrison *et al.* 2003a). It is possible that similar antibody mechanisms exist in *T.circumcincta* infection. However, in *T.circumcincta* infection, although IgA has been associated with growth retardation and inconsistently with reduced worm fecundity.(Halliday *et al.* 2007; Smith *et al.* 1984; Strain *et al.* 2002)**,** a recent study of IgA kinetics following infection would suggest that this antibody increases too late to be involved in early worm expulsion (Halliday *et al.* 2007).

IgE is likely to be involved in the hypersensitivity reaction, and its levels are closely correlated with those of mucosal mast cells, globule leucocytes, and in turn to FEC and worm burdens (Huntley *et al*. 2004).

Antibodies of the IgG class are produced during nematode infection, but it is not clear what role they play in immunity to *T.circumcincta* although IgG1 levels have been shown to relate closely to strongyle burden and FEC in animals selected for resistance (Bisset *et al*. 1996).

### 1.5.6 Potential Novel Effector Molecules

Work in mice has yielded much information on potential novel immune effector molecules, which may also play a role in the ovine host. A molecule of potential importance, induced by Th2 cytokines, is the goblet cell-specific protein RELM beta (resistin like molecule beta, also called FIZZ2) (Artis 2006a). Maximal RELMβ production occurred at the time of maximal Th2 cytokine levels and host protective immunity to three phylogenetically distinct GI nematodes in mice. RELMβ is essential for expulsion of *Nippostrongylus brasiliensis* in mice (Herbert *et al.* 2009). The potential effector role of RELMβ is thought to act by impairing the nematode's

sensation of the gut environment, thereby rendering it disorientated and unable to colonise its niche (Artis *et al.* 2004). Siat 4c (sialyltransferase 4c) is also up-regulated in mice following infection with *Trichinella spiralis* (Knight *et al*. 2004). Siat 4c may be involved in altering the acidity of mucosal glycoproteins that may affect parasite survival (Karlsson *et al*. 2000). Siat 4c expression has been shown to be significantly up-regulated in immune sheep challenged with *T.circumcincta*, compared to naïve, unchallenged sheep (French *et al*. unpublished data).

Intelectin is another intestinal protein that is upregulated in goblet cells of mice infected with *T. spiralis*. Interestingly, the intelectin 2 gene is absent from the parasite susceptible strain of mice, C57BL/10, but is significantly up-regulated in the resistant BALB/c mice (Pemberton *et al.* 2004a). The exact role of intelectin is yet to be established but recent work has shown that intelectin 2 mRNA is significantly up-regulated in ovine abomasal mucosa following challenge with *T.circumcincta* (French *et al*. 2008), and in airway goblet cells in vitro, following the application of the Th2 cytokine, IL-4 (French *et al*. 2007).

The work carried out in murine models of parasite infection has provided invaluable information through techniques such as gene knockout, which would be unachievable in the ovine host. However, more work is needed to establish the similarities in immune response between these two hosts, both in terms of the immune effector molecules discussed above, as well as a closer look at the sheep to determine if different novel proteins may have an important immune function unique to this host.

Our current understanding of immunity to *T.circumcincta* does not extend to the exact mechanisms involved in either the expulsion of the worms, nor their arrestment at the early L4 (EL-4) larval stage. This knowledge may be crucial if manipulation of these protective mechanisms is to be achieved through vaccination.

## *1.6 Genetics*

It has long been accepted that host genetics can influence the immune response to a parasite and hence affect the outcome of the host-parasite interaction (Dineen *et al.* 1978). It follows therefore, that host genetics will also influence the response to vaccination, a point which may become of prime importance in the future. Studies in Australia and New Zealand have identified a number of host immune functions thought to be under a strong genetic influence (Windon 1996), with particular reference to *Trichostrongylus spp.* infection. These include responses associated with superior antigen recognition, exhibited as parasite specific cellular and antibody responses, as well as effector responses (such as mast cells and circulating antibody) (Windon 1996).

These immune responses lead to a number of possible consequences for the host-parasite system. The manifestations of nematode resistance to *T.circumcincta* differ in their heritability, with adult worm length being the most heritable of resistance parameters (Stear *et al*. 2009). This is followed by the number of eggs produced by the adult worms, then the actual worm number itself. No genetic link has been made to the number of inhibited larvae (Stear *et al.* 2009).

Investigation into the genetic basis for resistance to the nematode *T.circumcincta* has repeatedly identified two regions of the ovine genome. These quantitative trait loci (qtl) are located on chromosome 20 (Davies *et al.* 2006; Paterson *et al.* 1998; Schwaiger *et al.* 1995) and chromosome 3 (Coltman *et al.* 2001; Sayers *et al.* 2005). Additional qtl for resistance to *T.colubriformis* infection have been identified on chromosmes 1, 6 (Dominik 2005) and 8 (Crawford *et al*. 2006). The mutations occurring at these locations have not been positively identified. However, it is thought that the mutation on chromosome 20 relates to the MHC class II DRB1 gene (Davies *et al.* 2006; Paterson *et al.* 1998; Schwaiger *et al.* 1995). Sheep heterozygous at the DRB1 locus are more resistant than homozygotes (Stear *et al.* 2005). The MHC class II genes influence parasite antigen recognition and so heterozygous animals will recognize a different parasite molecule repertoire

compared to the homozygotes (Stear *et al.* 2009). The mutation occurring on chromosome 3 is thought to involve the interferon γ gene (Coltman *et al.* 2001; Sayers *et al.* 2005). Interferon γ is a cytokine involved in selection of T helper cell responses, suppressing Th2 cell activity (Stear *et al.* 2009). It makes sense therefore that a polymorphism here could greatly influence the outcome of the immune response generated against an invading nematode. At this position, the F allele is thought to lead to susceptibility and the S allele to resistance (Stear *et al.* 2009). The aim of identifying these qtl would be to better understand the immune mechanisms leading to resistance, and, longer term, to aid in the breeding of disease resistant animals.

## *1.7 Anthelmintic Resistance*

Parasite resistance to anthelmintics is a rapidly increasing problem worldwide. Parasites of most species, including humans, horses, cattle and sheep, now show some degree of resistance. There is evidence that selection for resistance occurs more rapidly in tropical/sub-tropical regions compared to temperate areas, but nevertheless, resistance is becoming such a problem even in temperate areas that sustainable livestock production is not possible on some farms. Currently resistance is more of a problem in sheep and goats than in cattle, as resistant parasites seem to be selected quicker in these species (Waller 1997).

Resistant isolates of *T.circumcincta* have been reported worldwide. As this is the most important nematode species of sheep in the British Isles, emergence of so called "triple resistant" strains, resistant to all three major anthelmintic classes in common use, poses a major threat to the intensive sheep farming industry as we know it. In the UK, there are three classes of anthelmintic licensed for the treatment of *T.circumcincta* infection. These are the benzimidazoles (eg. fenbendazole), the macrocyclic lactones (the avermectins and milbemycins eg. ivermectin and moxidectin), and the imidazothiazoles (eg. levamisole).

The first case of benzimidazole resistant *T.circumcincta* was reported in England in 1982 (Britt 1982; Jackson & Coop 2000). By 1992, between 35 and 61% of sheep farms in Southern England were resistant to benzimidazoles (Hong *et al*. 1992), with *T.circumcincta* being the principal species involved. The most recent study found 64% of British farms tested are resistant to benzimidazoles (Bartley *et al*. 2003), historically the most widely used anthelmintic. Levamisole resistant *T.circumcincta* were also first reported in England, in 1996 (Hong *et al.* 1996). Resistance to all three available anthelmintic classes, including the avermectins, was first identified in goats (Coles *et al.* 1996), and then thereafter in commercial sheep flocks in Scotland (Bartley *et al.* 2004). There are now reported cases worldwide of triple resistant strains (Bartley *et al.* 2004; Wrigley *et al.* 2006).

### 1.7.1 Mechanisms of anthelmintic resistance

Drug resistance can occur through altered drug target, altered metabolism resulting in drug removal or inactivation, inhibited drug access to site of action or through amplification of genes responsible for counteracting the effects of the drug (Wolstenholme *et al.* 2004).

Benzimidazoles work by preventing tubulin polymerisation such that it cannot form microtublules (Wolstenholme *et al.* 2004). Benzimidazole resistance is related to mutations in the β-tubulin gene and these prevent drug binding. More than one polymorphism of this gene is thought be connected to benzimdiazole resistance (Prichard 2001). Kwa et al. first described a conserved mutation at amino acid 200 in β-tubulin isotype 1 (Kwa *et al.* 1994), and this is still considered to be the most important mutation in *H. contortus* (Wolstenholme *et al.* 2004). In *T.circumcincta*, a phenyalanine-tyrosine (Phe-Tyr) polymorphism exists at codon 167 (Silvestre & Cabaret 2002). This has also been identified in *H. contortus* (Prichard 2001).

Levamisole works by causing spastic paralysis of the worm through blocking transmission at the nematode neuromuscular junction. Morantel and pyrantel work in the same way. Resistance arises through changes in the target molecule (nicotinic

acetyolcholine receptors) of the nematode (Wolstenholme *et al.* 2004). Physiological and pharmacological evidence exists for this (Martin *et al.* 2004; Richmond & Jorgensen 1999; Trailovic *et al.* 2002), but the molecular basis of resistance is as yet unknown.

Avermectins and milbemycins act on ligand-gated channels, involved in nematode feeding, reproduction and motility (Feng *et al.* 2002; Yates *et al.* 2003). Mechanisms of resistance to this anthelmintic family are not clear and may be multiple. There is evidence for mutation in the genes determining the glutamate- (GluCl) and GABA-gated chloride (GABACl) channels (Blackhall *et al.* 1998b; Blackhall *et al.* 2003; Njue *et al.* 2004) contributing to the resistant trait and also evidence for the overexpression of P-glycoproteins (Blackhall *et al.* 1998a; Kerboeuf *et al.* 2003), which leads to faster clearance of the drug from the worm.

### 1.7.2 Risk factors for developing resistance

#### 1.7.2.1 Over-use of anthelmintics

It is thought that resistant alleles occur not through forced mutation, but through natural selection of genotypes (pre-existing mutations) already present in a low frequency in the population – this is termed a pre-adaptive phenomenon (Jackson & Coop 2000). It follows then that resistant alleles become dominant quicker where there is increased use of anthelmintic, hence exerting a greater selective advantage on the nematodes carrying resistant genes. This could explain why resistant nematodes appear to be slower to develop in cattle, as these animals are generally subjected to less frequent anthelmintic treatments.

#### 1.7.2.2 Under-dosing

Conversely, resistant nematodes arise relatively swiftly in goats – a combination of the need for frequent anthelmintic treatment in this species (Jackson & Coop 2000) and rapid drug metabolism resulting in effective under-dosing. Under-dosing is

another factor which can accelerate the development of resistance. It is thought that nematodes heterozygous for the resistance gene may survive a sub-optimal dose of anthelmintic and in turn this will increase the frequency of the resistant allele in the population (Jackson & Coop 2000).

### 1.7.3 Measures to prolong the life of anthelmintics

#### 1.7.3.1 Population in refugia

Recent guidelines have been produced to help slow the spread of resistant alleles and prolong the useful lives of available anthelmintics. Research has been conducted worldwide into this topic and, in Europe, the PARAsol consortium has had a significant part to play. Much of the advice is geared towards maintaining a significant parasite population in refugia. That is, parasites which are not exposed to drugs but which are allowed to complete their lifecycles. Largely this will refer to parasites in the free-living stage, on pasture (Kenyon *et al.* 2009). Management advice has moved away from the traditional "dose and move" strategy, towards "move and dose" or leave on current pasture for a week or so prior to moving to clean pasture, in order to maintain this population in refugia. Another source of refugia are particular lifecycle stages which may not be susceptible to certain anthelmintics. For example hypobiotic larvae (Fleming *et al*. 2006) and immature larvae which are not susceptible to Levamisole (Grimshaw *et al.* 1996; Kenyon *et al.* 2009).

#### 1.7.3.2 Targeted selective treatments

There has been a lot of recent interest in maintaining a parasite population in refugia through the implementation of targeted selective treatments. This means that certain individuals are not treated at all and so continue to contaminate pasture with anthelmintic susceptible worms. Targeted selective treatment is based on the fact that nematode numbers in a population have a skewed distribution so that a small

number of individuals carry the majority of the parasite burden. The parameters which are chosen to dictate which animals are targeted for selective treatment is perhaps the most difficult to define and varies depending on host species, nematode species and commercial purpose of the host (Greer *et al.* 2009; Kenyon *et al.* 2009; Stafford *et al.* 2009).

#### 1.7.3.3 Annual rotation of anthelmintics

For many years now, the annual rotation of anthelmintics has been used by many in an attempt to slow the development of resistance. However the value of this strategy has never been demonstrated in the field. Instead, modelling studies have suggested that combination treatments are better at delaying the onset of resistance (Barnes *et al.* 1995). No such products are licensed in the UK, but are available in Australia and New Zealand. The use of combination products when resistance alleles are already high may be efficacious in the short term, but are unlikely to slow down the selection of resistant nematodes (Hennessy 1997).

## *1.8 Alternative control*

### 1.8.1 Pasture Management

For years, grazing management has played a key role in the control of parasitic gastroenteritis on many UK farms. It has the advantage that it reduces the reliance on anthelmintics for profitable livestock production.

The simplest means of reducing the number of infective larvae on pasture would be to reduce stocking density. As well as reducing pasture contamination, animals have greater choice of where to graze and will naturally avoid grazing near faeces and the associated nematode larvae (Stromberg 1997). This style of grazing management is clearly not suited to many lowland UK farms where intensive livestock rearing is paramount to profit, but may be appropriate for rougher grazing such as is found in extensive hill farms.

Alternating pasture use is another means of grazing management designed to aid the control of nematode infection. This may be either through grazing with a different species, for example cattle one year, sheep the next, or by adding in a year where the pasture is used to grow crop and is not grazed at all. Both of these methods help to reduce pasture contamination for the following grazing season. There are few nematode species which infect both sheep and cattle but those that do, such as *Trichostrongylus axei,* tend to be less pathogenic than the species specific mixed infections (Stear *et al*. 2003). Co-grazing sheep and cattle on the same pasture effectively reduces the stocking density of each species. Because neither species is susceptible to nematodes from the other, this practice dilutes the infective larvae available on pasture, and hence the challenge to the grazing animals. However, this system is not suited to all farming systems, especially as many farms currently are committed to a single enterprise and so have neither the facilities nor the financial incentive to adopt this approach.

Alternatively animals can be grazed in a paddock rotation system whereby they move frequently throughout the summer to "clean" pasture, again reducing the number of infective L3 they are exposed to. It is reliant upon the numbers of infective larvae on pasture having reduced by the time the animals return to the original pastures. This will be more successful in some years than others as climate can have a significant impact on larvae survival (Callinan & Arundel 1982). This type of system has a high requirement for grazing land but can be incorporated into some farming systems by the use of silage aftermath, for example.

The strategic use of anthelmintic prior to movement to fresh pasture has been widely used in order to preserve the "clean" status of pasture. Until recently, this "dose and move" strategy was the gold standard and was indeed effective at reducing pasture contamination. However, with the emergence of anthelmintic resistant nematodes, this strategy becomes detrimental and encourages the selection of resistant strains (Sutherland *et al.* 2002). In addition, the advice is no longer to dose all animals, but rather to leave a susceptible population of worms in refugia (as discussed in section 1.7.3.1), through the use of targeted selective treatment programmes (Greer *et al.*

2009) or part flock treatments. However, the uptake of these labour intensive programmes is likely to be slow in some parts of the world (Cabaret *et al.* 2009; Cringoli *et al.* 2009) and the benefits still have to be weighed against production losses caused by nematode infection.

The Sustainable Control of Parasites in Sheep (SCOPS) is a UK industry led initiative which attempts to address, among other things, the issue of pasture management in the face of emerging anthelmintic resistance. It gives advice for veterinary surgeons and farmers on best practice for the control of gastrointestinal parasites, whilst reducing the selective pressures for resistant anthelmintic strains.

### 1.8.2 Nutritional Supplementation

Nutrition is known to affect the impact that parasite infection has on an animal. Coop and Kyriazakis (Coop & Kyriazakis 2001) summarised that this effect could manifest in one of three ways : resilience, resistance and through direct adverse effects on the parasites themselves.

#### 1.8.2.1 Resilience

Resilience is the ability of the host to perform under adverse conditions of parasitism. Infection with *T.circumcincta* results in a metabolic protein deficiency through pathology associated with protein loss and also through allocation of protein to immune functions (Coop *et al.* 1977; Coop *et al.* 1985). By providing more protein, this relative protein deficiency can be overcome and production parameters, such as live-weight gain, wool growth and carcass quality, can be improved. This has been shown to be the case in both controlled trials (Abbott *et al.* 1985; van Houtert *et al.* 1995) and in parasitised animals in a natural grazing setting (van Houtert *et al.* 1995). Encouragingly the use of urea, a source of rumen degradable nitrogen, which is an inexpensive source of nitrogen compared to the higher quality undegradable proteins, also has positive effects on resilience (Knox & Steel 1999;

Wallace *et al.* 1998). This makes the prospect of protein supplementation more attractive, given the small financial margins involved in livestock production.

#### 1.8.2.2 Resistance

Resistance is the ability of the host to overcome infection and can be measured by parasitological parameters such as fecundity, worm burdens and the persistence of infection. High protein diets are likely to affect immunity through distribution of nutrients, with greater resources being available for immune responses. This is particularly true for growing animals where protein allocation to growth takes priority over immune functions (Coop & Kyriazakis 2001). Enhanced immune function following protein supplementation has been demonstrated against *T.circumcincta* (Coop *et al.* 1995), *H.contortus* (Strain & Stear 2001), and *T.colubriformis* (Kambara *et al*. 1993). Supplementing protein has an effect on the immune effector mechanisms, directly affecting immune cell numbers (eosinophils, globule leukocytes and mast cells) (Coop *et al.* 1995; van Houtert *et al.* 1995). Supplementary feeding also has a role in reducing the peri-parturient rise in egg production of pregnant and lactating ewes (Houdijk *et al.* 2001a;Houdijk *et al.* 2001b). On certain farms, depending upon soil composition, supplementation with trace elements may also be beneficial. Iron, zinc, molybdenum and copper have all been shown to influence host resistance to nematode infection (Waller & Thamsborg 2004).

#### 1.8.2.3 Bioactive forages

Some crops, through the action of secondary plant metabolites, can have a direct adverse effect on the nematodes. These can be termed bioactive forages and include plants containing phenolic secondary metabolites, tannins. Tannins have been implicated as important dietary components potentially involved in reducing development of infective nematode larvae (Niezen *et al*. 2002). The mechanism of action of tannins is unclear. It may be through a direct effect on eggs and subsequent

larvae, as these compounds are not absorbed from the GI tract, and so are unlikely to affect immunity (Waller & Thamsborg 2004). Or, they may actually affect resilience and resistance through an indirect improvement in protein nutrition by protecting protein in the rumen such that it passes through undegraded (Waghorn *et al*. 1987).

Chicory (Circhorium intybus) has been shown in an organic farming system to reduce lamb faecal egg counts and improve liveweight gains to levels comparable with those lambs reared by anthelmintic treated ewes (Athanasiadou *et al.* 2007). Reduced faecal egg counts in lambs and improved liveweight gains were also evident in a separate study looking at the combined effects of maternal nutrition and subsequent grazing of lambs on chicory pasture versus grass/clover pastures (Kidane *et al.* 2009). It has been suggested that animals grazing chicory have an improved immune response to incoming larvae, as measured by mucosal mast cell and globule leukocyte counts (Tzamaloukas *et al.* 2006). Another study has suggested that the sward structure of the chicory plant may actually affect larval migration, survival or development on pasture and in this way reduce exposure of susceptible sheep to infective larvae (Marley *et al.* 2006).

Despite the success of supplementary feeding in nematode control, it is not widely practiced. This is due to the costs of additional feeds overwhelming what, for most, is a narrow profit margin in most livestock production systems. As long as they remain efficacious, the use of anthelmintics makes more financial sense. Bioactive forages may be more realistic, but again the benefits the crops bring through reduced parasitism must outweigh the costs associated with reduced feed intake and digestibility. The viability of the crops in certain climatic regions will also be an issue. Although not likely to achieve absolute control when used alone, nutrition may be an important adjunct to traditional control methods and reduce the dependence upon anthelmintics.

### 1.8.3 Biological Control

The possibility of biological control has also been investigated. Nematodes, like all other groups of organisms, have organisms that prey on them (Waller & Faedo 1996). Biological control involves the manipulation of these natural predators in order to control the parasite population.

The nematode-destroying microfungus, *Duddingtonia flagrans*, has been the subject of experimentation due to its ability to survive passage through the ruminant GI tract (Larsen 2006). As a result it is deposited in the faecal pat along with the nematode eggs. The fungus can reduce the number of infective larvae on pasture and hence the exposure of susceptible animals (Waller & Thamsborg 2004). In coprocultures and plot studies, it has been shown to have an efficacy exceeding 90% for cattle parasites, and 70% for sheep parasites (Ketzis *et al.* 2006). It has recently shown some potential when used in field trials in Australia (Larsen 2006). However, its effects are variable and strongly dependent on favourable climatic conditions for spore survival (Epe *et al*. 2009)

The concern with any biological control method would be an inadvertent effect on a non-target species, which could greatly influence the ecosystem. In this instance, *D. flagrans* has been shown to be non-pathogenic to soil dwelling, non-parasitic nematodes (Knox *et al.* 2002). A major hurdle to be overcome in the use of *D.flagrans* is the need for daily in-feed administration of the fungal spores (Ketzis *et al.* 2006; Larsen 2006). For many farming practices, this would not be feasible for both financial and logistical reasons. Again, this alternative control strategy may prove a useful addition to pasture management and anthelmintic control, but is unlikely to be effective enough to eliminate the production losses associated with *T.circumcincta* infection on its own.

### 1.8.4 Selective breeding for resistant animals

As our knowledge of the genetic basis of resistance widens, the possibility of selective breeding for nematode resistance on a commercial scale becomes more of a reality. Selection experiments have been taking place in Australia and New Zealand for a number of years (Windon 1996). These highlight the importance of investigating genetic resistance at the animal level, as well as at the molecular level. Windon (1996) summarised four principal aims of these selective breeding programmes: 1 - to assess genetic correlation between nematode resistance and important production traits; 2 - to determine resistance mechanisms which are under genetic control; 3 - to identify genetic and phenotypic markers which could be used to select for resistance in a commercial breeding programme; 4 - to determine if resistance to other pathogens, or different nematode species, is linked to resistance to the nematode of interest (Windon 1996). Selection for resistance may not be straightforward as breeding for low FEC is associated with reduced fleece weight, reduced milk production and, of most importance for the UK industry, reduced post-weaning lamb liveweight gains (Greer 2008).

Although commercial testing and breeding for nematode resistance is still at an early stage, the concept of selective breeding for disease resistance is not a new one. Selective breeding in UK sheep herds has been successfully implemented in the control of the prion disease Scrapie. This has been through the genotyping of the PrP gene in breeding rams, introduced through The National Scrapie Plan in 2001. This scheme demonstrates the potential for selective breeding programmes aimed at improving disease resistance.

## *1.9 Vaccination*

Vaccination is a possible alternative for control of parasitic nematodes in ruminants. The prospect of successful control through vaccination is made more likely due to the immunity that is known to build up over time as animals are continuously exposed to the parasite. In vaccine design it is important to consider the qualities of

a livestock vaccine that will determine whether or not it is marketable, and not only its efficacy in controlling a particular parasite.

A successful nematode vaccine would need to rival the alternative (anthelmintics), both in terms of cost and in spectrum of activity across nematode species (although niche markets may exist in the Southern hemisphere where *Haemonchus* spp. predominates and in this instance a vaccine directed against a single nematode species may be profitable (Knox 2000)). In addition, it would need to be administered in such a manner, and with a frequency, that conformed to existing management practices. The efficacy of a vaccine needs to be carefully considered - enough to prevent overt disease and gradually reduce pasture build up over time, but not so much as to avoid the natural immunity developing as a result of exposure (Knox 2000). Generally the desire will not be to vaccinate periodically throughout the animal's life, but only when it is young.

In developing vaccines, the required efficacy needs to be defined. In the case of a parasite vaccine, the level of protection required will vary depending on whether the vaccine is a stand-alone control procedure or is applied in conjunction with management procedures which may reduce exposure to the parasite. Minimising or eliminating the clinical consequences of infection should be a goal (Knox & Redmond 2006). In general terms, the "performance" requirements for a vaccine are defined on the basis of epidemiological data and mathematical modelling (Knox & Redmond 2006). However, user perception is likely to be very influential. For example, livestock producers are likely to compare a vaccine with control achieved with anthelmintic drugs and ectoparasiticides, which, when first introduced, approach 100% efficacy. However, it is unlikely that anti-parasite vaccines will attain this level of efficacy and computer modelling of population dynamics suggest it is not essential (Barnes *et al.* 1995). Therefore, the introduction of a vaccine is going to require a sustained educational effort targeting veterinary practitioners and agricultural advisors.

Despite extensive research both in the livestock sector and in human medicine, very few helminth vaccines are currently commercially available. Vaccination has been used for many years in the successful control of lungworm in cattle (*Dictyocaulus viviparous*) (Jarrett *et al*. 1960) and sheep (*Dictyocaulus filaria*) (Dhar & Sharma 1981). Both these vaccinations use whole, irradiated L3 larvae. A similar approach has been tried for the gastrointestinal nematodes *Haemonchus contortus, Trichostrongylus colubriformis* and *Teladorsagia circumcincta* (Dineen *et al.* 1978; Jarrett *et al.* 1961; Smith *et al.* 1982; Smith & Christie 1978). Irradiated *T.circumcincta* larvae were found to be less immunogenic when compared to *H.contortus* or *T.colubriformis* (Smith *et al.* 1982). Using whole irradiated larval vaccines has the disadvantage of requiring a prolific source of worms, and, since *T.circumcincta* cannot be grown in culture, this means the sacrifice of many donor sheep.

Another approach to nematode vaccine development has been through the identification and isolation of parasite antigens. Parasite antigens can be classified depending on their source of origin. They can either be found within the parasite tissues (somatic), on the surface of the parasite (cuticular) or they can be released by the parasite. Considerable progress has been made in identifying useful protective antigens in *H. contortus*, particularly antigens expressed on the surface of the intestine in the blood-feeding stages which are accessible to attack by vaccine-induced systemic antibody responses. The approach of targeting intestinal proteins in non-blood feeders such as *T. circumcincta* and *O. ostertagi* has been less effective.

A cysteine protease-enriched excretory/secretory (ES) fraction from adult *H. contortus* induced significant protection against infection in lambs (Bakker *et al*. 2004) and an equivalent preparation from adult *Ostertagia ostertagi* reduced faecal egg output in calves by 60% (Geldhof *et al*. 2002). The major protein component of this fraction is also an activation-associated secreted protein (ASP) homologue and recent work has shown that this ASP and the cysteine proteases are both protective in their own right (Meyvis *et al*. 2007). A homologue of the polyprotein allergen proteins, found in adult and L4 *O. ostertagi* ES, also stimulates significant protective

immunity, 60% reductions in faecal egg output, in recipient calves (Vercauteren *et al*. 2004). It is worth emphasising that reductions in faecal egg output of around 60% under natural infection conditions would be sufficient to substantially protect calves during the entire first grazing season (Claerebout *et al.* 2003).

To date, no effective vaccine antigens have been reported for control of *T. circumcincta.* For non-blood feeders, such as *T.circumcincta*, systemic antibody responses are unlikely to confer adequate immunity and local mucosal immune responses are likely to be necessary. Hence it is not only antigen selection which is important, but the way in which that antigen is delivered in order to induce an appropriate protective immune response. This includes appropriate selection of adjuvant, as well as potential use of such technologies as antigen delivery using vectors, such as salmonella (Chatfield *et al*. 1995) or vaccinia. Antigen delivery in association with cytokines, designed to stimulate the most appropriate immune response, or DNA vaccination are other possibilities to be explored in the future (Knox *et al*. 2001).

## *1.10 Aims and Objectives*

Current research into the nematode parasite of sheep, *T.circumcincta*, is aimed at vaccination. To date, this goal has proven to be unachievable, but a greater understanding of the mechanisms involved in natural immunity may aid in vaccine design. In particular, further information on effector responses within the abomasal mucosa is desirable. Towards this end, this thesis used proteomics to study a well established infection model, involving the analysis of gastric lymph from sheep infected with *T.circumcincta*. Using lymph to study the local response to infection has the advantage that individuals can be followed temporally throughout the infection period, a position clearly not possible with post-mortem sampling. Previous studies showing that locally expressed proteins such as pepsinogen are detected in lymph following induction of the leak lesion suggested that novel mucosal effector molecules could also be detected in this way. This has afforded an initial global overview of the gastric lymph proteome and highlighted specific

proteins such as the acute phase proteins and gelsolin for further study. In view of the central importance of the leak lesion to these studies, the tight junction proteins were also studied as a possible mechanism by which the "leak lesion" phenomenon occurs.

By methods described above, the aim of this thesis is to contribute to the available knowledge regarding the immunological and pathological processes occurring in the abomasum. These innate, local responses are potentially crucial in contributing to immunity, and as such may be key considerations in vaccine design that aims to promote the effector responses involved in natural immunity to parasites.

# 2 Chapter 2: Materials and Methods

## *2.1 Animal experiments*

Gastric lymph was collected following cannulation of the common gastric lymph duct. In the ovine subject, this lymph duct drains all four ruminant stomachs (Smith *et al.* 1981). Despite this, it is still a suitable medium for studying the local reactions within the abomasum, as the other three stomachs (reticulum, rumen, omasum) are all relatively immunologically inert and so make minimal contributions to the changes occurring within the gastric lymph (Smith *et al.* 1981).

### 2.1.1 Surgical procedure for lymph duct cannulation

The method used for this was based on an established procedure (Smith *et al*. 1981) with some minor alterations relating mainly to advances in anaesthetic and chemotherapeutic agents. General anaesthesia was induced using intravenous Thiopentone and maintained using gaseous Isoflurane. The surgical approach was via a right flank incision, immediately caudal to the last rib. The abomasum was exteriorised and 1ml of Evan's blue injected sub-serosally into multiple sites on the wall of the abomasum. Following replacement of the abomasum into the abdominal cavity, the efferent arm of the common gastric lymph duct could be visualised, by means of dye flow, in the region of the epiploic foramen. Following blunt dissection around the caudal vena cava to improve exposure of the duct, a tight ligature of 3/0 Ivory Mersilk (Ethicon) was placed down-stream i.e. caudally from the intended incision site, to cause the duct to swell. A loose ligature was place upstream of the intended incision site before the duct was incised. An SV 45 cannula (Dural Medical Tubing) was fed into the duct before being anchored in place by the tightening of the upstream ligature and re-tying of the downstream ligature. The cannula was exteriorised through a separate incision in the body wall, distinct from the laparotomy wound.

In order to allow re-infusion of the lymph back into the circulation, a 12 G Intraflon Catheter was inserted into the caudal vena cava at the level of the lymphatic

cannulation. The end of the catheter was attached to an IV fluid giving set (Aquapharm) and continued through the body wall, again at a separate incision distinct from the laparotomy wound. The abdominal wound was closed by means of vicryl sutures (Straight/cutting/2M Vicryl, Ethicon) in the transverse and oblique abdominal muscles and 4 metric Braun Dacrofil sutures in the skin.

Every animal was given a NSAID painkiller (Meloxicam, Metacam™, Boehringer Ingelheim) and antibiotic (amoxycillin-clavulonic acid, Synulox™, Pfizer) immediately prior to recovery from anaesthesia. The dose of these drugs was calculated as per data sheet recommendations for the weight of the individual sheep.

Each animal was fitted with a harness, which supported a heparinised lymph collection bag. A sample of lymph from the bag and a fresh flowing sample were collected every 24 hours. The remainder of the lymph in the collection bag was then re-infused back into the animal via the catheter placed in the caudal vena cava. Blood samples were also collected at 24 hour intervals, either from the in-dwelling catheter or via jugular veno-puncture.

Blood and lymph samples were centrifuged (2000rpm/20min) to yield separate plasma and cell fractions. The samples were stored at –20$^{o}$C until needed.

### 2.1.2 Experimental Design:

This experiment had equal numbers of cannulated and non-cannulated animals.

#### 2.1.2.1 Cannulated animals

Cross-bred lambs (n=10) were born and reared to 10 months of age indoors in a worm-free environment. The animals fell into two categories: immune challenged, or naïve, primary infection animals.

The immune challenged animals were trickle infected with 2,000 L3 (the infective stage) *T.circumcincta* larvae, three times weekly for 8 weeks, in order to induce a degree of immunity. They were then treated with anthelmintic (Levamisole at the recommended dose rate) 7 days prior to experimental challenge. The common

gastric lymph duct of these animals was then surgically cannulated, and lymph and blood were collected daily throughout the experimental period (see below). Approximately 3 days after cannulation, sheep were given an oral challenge dose of 50,000 L3 *T.circumcincta* using a levamisole susceptible ovine strain maintained at Moredun Research Institute for many years (CVL – MTC12). Sheep were killed on day 10 after challenge and abomasal worm counts were performed using the established method (Coop *et al.* 1977), to confirm successful infection.

The primary infection animals were treated in exactly the same way from the point of anthelmintic treatment (but did not receive the trickle infection). Faecal egg counts were performed on the previously naïve sheep from day 18 of infection to confirm patency of the nematode infection. Sheep were killed at day 21 and abomasal worm counts were performed.

#### 2.1.2.2 Non –cannulated animals

All other samples (tissues and blood), were collected from non-cannulated animals. These experiments ran in parallel with the cannulated animal experiments. The non-cannulated animals were of the same origin and were treated exactly as for the immune challenged and naïve primary infection animals above. The only difference being that in these experiments the animals did not undergo cannulation surgery, and therefore lymph could not be collected for analysis. Instead, blood samples were collected from these animals by jugular venopuncture three times weekly for a 21 day period from the day of challenge (for acute phase protein analysis, Chapter 4). In addition to the experimental group (n=10) identified above, tissues from equivalently treated sheep from a larger scale initiative within the Institute were available at various time points after infection. Tissue samples were collected from these sheep at various time-points (n=6 at days 0, 2, 5, 10 and 21) during the infection period. It is these post-mortem samples which were used for subsequent PCR experiments in Chapters 4 and 5 and for immunofluorescence studies in Chapter 6.

## *2.2 Parasitological procedures*

### 2.2.1 Faecal Egg Counts

Faecal egg counts were carried out in all sheep undergoing a primary infection, and in all non-cannulated animals following oral infection with *T. circumcincta* L3. This was to ensure that patent infections had established. FECs in cannulated animals were carried out by Lois Parker and Stephen Smith of the Moredun Research Institute. Briefly, 10ml of water was added per 10 grams of fresh faeces and the mixture emulsified. A 10ml sub-sample was then passed through a 1mm sieve and the retentate washed with approximately 5ml of water. The filtrate was then spun for 2 minutes at 1000rpm and the supernatant removed by means of a vacuum line. 10ml of saturated sodium chloride solution was added to the faecal pellet, and the solid content gently resuspended. The mixture was centrifuged again at 1000rpm for 2 minutes in Beckman polyallomer centrifuge tubes. The high salt content of the solution causes the eggs to float on the top of the liquid, while all solid debris is pelleted at the bottom of the centrifuge tube. Using artery forceps, the top of the tube was clamped just below the meniscus and the upper contents transferred to a cuvette. The upper portion of the tube was rinsed with saturated salt solution and this too was transferred to the cuvette. The cuvette was filled until there was a small positive meniscus and then a lid was applied from the side to prevent air bubbles. The cuvette was placed under a microscope and the eggs present on the upper surface were counted with the aid of a graticulate square.

### 2.2.2 Abomasal worm counts

Abomasal worm counts were carried out by Stephen Smith and Lois Parker at the Moredun Research Institute. The method used was as published by Coop et al. (Coop *et al.* 1977). In brief, at post-mortem the abomasum was ligated and excised along the greater curvature. The contents were collected and the surface of the abomasum washed in 1% sodium bicarbonate. Washings and abomasal contents were allowed to sediment at 4$^{o}$C. The abomasum was then digested in 1% pepsin with HCl for 4 hours. The solution was neutralised and the sediment preserved in

formalin. Worms in aliquots of washings and digests were counted and classified according to sex and stage of development.

## *2.3 Protein Determination*

Protein determination was carried out using a commercially prepared kit – Pierce BCA Protein Assay Kit. This kit follows a two-step protocol. Step 1 involves the chelation of copper with protein, resulting in the reduction of $Cu^{2+}$ to $Cu^{1+}$. In step 2, BCA is added, which reacts with the $Cu^{1+}$ and results in the formation of a purple coloured product. This BCA/copper complex shows a strong linear absorbance at 562nm proportional to increasing protein concentration. Protein concentration was calculated based on a standard curve of UV absorbance at 562nm.

## *2.4 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)*

### 2.4.1 1-dimensional (1D) SDS PAGE

This technique separates proteins based on their molecular weight. By mixing the proteins in an excess of SDS, the proteins become negatively charged. In the presence of an externally applied electric charge the SDS-coated proteins migrate towards the anode. The rate of migration through the gel is determined by molecular weight. SDS-PAGE gels can be run reduced or non-reduced. When the samples are reduced by the addition of e.g. dithiothreitol (DTT) or 2-mercaptoethanol to the sample buffer, disulphide bonds are eliminated and proteins migrate as individual subunits. The reducing agent is not included for non-reducing fractionation and disulphide bonds are retained. Both reducing and non-reducing gels have been used in this thesis.

The sample (e.g. lymph plasma, or abomasal protein extract) was added to an equal volume of sample buffer (either reducing or non-reducing buffer). The mixture was incubated at 100$^{o}$C for 5 minutes (to ensure complete denaturation (reducing only)).

Separating gels (10%) were prepared as follows: 2.5ml of 1.5M Tris-HCl, pH8.8 was added to 4.02ml of $dH_2O$, 3.33ml of acrylamide-bisacrylamide stock (30%w/v) (Protogel), 100µl (10%w/v) SDS stock, 100µl of 10% ammonium persulfate, and 10µl of TEMED (Tetramethylethylenediamine). The mixture was gently inverted in a universal container to mix. 3.5ml of the mixture was pipetted into the gel casting plates (Bio-Rad mini-protean system) and $dH_20$ was overlayered. The gel was left to set for 20 minutes before pouring off the excess water. The stacking gel was then added. The solution comprised 2.5ml of 0.5M Tris-HCl, pH6.8 to 6.1ml of $dH_20$, 1.3ml of acrylamide-bisacrylamide stock (30%w/v) (Protogel), 100µl of 10% (w/v) SDS and 10µl of TEMED. This mixture was added on top of the separating gel until the gel space was full. A comb (either 10 or 15 well) was inserted into the liquid stacking gel. The stacking gel was left to set for 20 minutes.

In addition to these freshly prepared gels, precast gels were also used. For those gels (BioRad (USA), 10 or 15 well gradient gels (4-15%)), the gel was set up in the electrophoresis apparatus according to the manufacturer's instructions. (Gradient gels, formed through a continuously changing acrylamide concentration in the polymerisation solution, leading to a pore gradient, give better separation of large and small proteins on the same gel).

Following the addition of running buffer to the apparatus, each sample was applied to a well (approx 10-15 µl total sample, containing approximately, 5ug of protein, unless otherwise detailed). The apparatus was connected to a power supply and run at 200 V for approximately 40 minutes or until the dye front reached the bottom of the gel.

After electrophoresis, gels were stained in Coomasie blue stain (0.025% Brilliant Blue R(Sigma)), 40% methanol, 10% glacial acetic acid), for 60 minutes and a wash in distilled $H_2O$. De-staining was carried out by immersing the gel in 10% acetic

acid (v/v)/20% methanol (v/v) changed at 30 minute intervals until the protein bands were visible against a clear background.

### 2.4.2 2-DE (2-Dimensional gel electrophoresis)

2-Dimensional separation separates proteins firstly through differences in charge, and then in molecular weight. Charge based separation takes the form of isoelectric focussing (IEF). The net charge of a molecule depends on the sum of all the negative and positive charges attributable to the amino acid side chains. The mechanism of isoelectric focusing is based on the principle that, when a group of proteins is placed in a certain pH value, different proteins will have different net charges. In the presence of a stable pH gradient, the proteins will migrate towards the cathode or anode, depending on whether they have a positive or negative net charge, until they reach a pH where they are isoelectric. The isolectric point (pI), which is unique to that protein, depends on its composition and is the point where the molecule carries no net charge and, as a result, ceases to migrate.

The second dimension involves separation based on molecular weight, similar to 1-D SDS PAGE. In 2–DE, the separated proteins take the form of spots, as opposed to bands. For these analyses, proteins were concentrated by acetone precipitation prior to analysis. Acetone, stored at –20$^{o}$C, was added to the sample at a ratio of 4:1. The sample tubes were vortexed before storage at –20$^{o}$C overnight. The tubes were then centrifuged at 3000 rpm (RCF 2560g) for 45 minutes and the supernatant decanted off. The remaining protein pellet was then re-suspended in the appropriate buffer for further processing.

Acetone precipitated protein (equivalent of 80µl of start lymph, depleted of IgG and albumin, see section 2.5) was re-suspended in 350 µl of rehydration buffer (2% CHAPS / 8 M urea, ampholine mixture (pH 3-10, non-linear; 0.5%v/v; Amersham Biosciences) and DTT (60 mM)). The urea component solubilises and denatures proteins, unfolding them to expose internal ionisable amino acids. CHAPS, a detergent, helps solubilise hydrophobic proteins and minimises protein aggregation.

Dithiothreitol (DTT) reduces the disulphide bonds in the protein, allowing them to unfold completely. The ampholyte mixture, also called IPG buffer (Amersham Biosciences) is specially selected based on the pH range over which separation is to be achieved. It improves separation by enhancing protein solubility and produces more uniform conductivity across the pH gradient without disturbing IEF or affecting the shape of the gradient.

This mixture was then applied to immobilized pH gradient (IPG) strips (18cm pH 3-10 non-linear) (Amersham Biosciences); and left to rehydrate overnight. Following rehydration, the strips were prepared with electrodes and placed on the focussing apparatus (IPGPhor, Amersham Biosciences) and the proteins focussed for a total of 35,000 volt hours (VHr) according to the following focussing schedule: 100V 30 minutes; 200V 30 minutes; 500V 30 minutes; 2000V 30 minutes; 4000V until 13,000 total volt hours (TVh) and 8000V until 35,000 TVh. The focussed strips were stored at –80$^{o}$C until required for the second dimension separation.

Before transfer of the proteins from the strips to the 18cm, gradient gels (12-14%) (Amersham Biosciences), the rehydrated IPG strips were equilibrated in buffer (1M Tris HCl (pH 8.8), 6M urea, 30 % v/v glycerol, 2%v/v SDS and 0.002%w/v bromophenol blue) containing DTT (10 mg/ml) for 15 minutes, followed by 15 minutes in equilibration buffer containing iodoacetamide (25 mg/ml). The IPG strips were then applied to the gel on the Multiphor 2 (Amersham Biosciences) apparatus and the proteins were separated by SDS-PAGE. The gels were fixed overnight in a 45% (v/v) methanol / 1% (v/v) acetic acid mixture, and then stained in Colloidal Coomassie Blue stain (Proteomic Solutions) for 24 hours (or silver stained – see section 2.9).

## *2.5 Protein depletions prior to proteomic analyses of lymph and plasma*

Albumin and immunoglobulins are dominant proteins in plasma and lymph and, as such, have the potential to mask less prominent proteins in downstream proteomic

analyses. Simple methods were available to deplete these proteins prior to proteomics being conducted.

### 2.5.1 Albumin depletion of lymph and plasma

Albumin was removed using commercially available spin columns (IgY Protein partitioning Kit; Beckman Coulter) containing chicken IgY antibody generated against bovine serum albumin, in order to make the less abundant proteins relatively more concentrated in the remaining lymph. Each column had the capacity to bind albumin from 40 µl of lymph plasma (or 20 µl of blood plasma). Samples were diluted to a total volume of 500 µl with TBS (10mM Tris-HCl, pH 7.4, 150mM NaCl), then processed according to the manufacturer's instructions. Columns were regenerated using 0.1M glycine-HCl according to the instructions, and eluted albumin fractions were neutralised with 1M Tris-HCl, pH 8.0 and stored at -80ºC. Albumin depleted samples were then depleted of IgG (see below).

### 2.5.2 Immunoglobulin G (IgG) depletion of lymph and plasma

IgG depletion was achieved using home-made spin columns containing Protein G Sepharose (Sigma Aldrich). Protein G is a group G streptococci protein which binds strongly to the Fc region and weakly to the Fab region of IgG (Bjorck & Kronvall 1984). Each column contained 0.5 ml of Protein G Sepharose, suspended in aqueous ethanol. Each column run had the capacity to bind IgG from 80µl lymph plasma.

Prior to use, the spin column was washed with TBS (3 x 500 µl). Samples (80µl of albumin depleted lymph diluted to 500 µl with TBS) were incubated for 15 minutes on the column, while rotating end-to-end, then centrifuged (400g for 30 seconds) to elute the IgG depleted sample. Samples were then precipitated with cold acetone prior to Iso Electric Focussing (IEF). Columns were regenerated by washing in TBS (3 x 500 µl), followed by elution of the bound immunoglobulin from the column by two, 3 minute incubations in 0.1M glycine, pH 2.5 (500 µl). The column was then incubated (5 minutes) in 0.1M Tris-HCl, pH8.0 (500 µl), and then re-equilibrated in TBS. Eluted IgG fractions were neutralised with 1M Tris-HCl, pH 8.0, and stored at –80$^{o}$C.

## *2.6 LC-ESI-MS/MS of bound fractions from depletion columns*

This technique was carried out to determine which, if any, proteins were being inadvertently removed by the depletion columns. It involved one-dimensional separation of the protein mixture, followed by subdivision of the gel into segments, trypsin treatment and liquid chromatography and then mass spectrometry of the separated peptides. This analysis was carried out on my behalf by Neil Inglis of the Proteomics Facility at Moredun Research Institute. The sample used was from an unchallenged, naïve animal. The procedures used are outlined below.

### 2.6.1 Primary sample preparation

Approximately 10µg of total protein eluted from each of the columns, following standard lymph depletion (as detailed above), was separated on a 1-D SDS PAGE gel (Ready Gel Precast Gels, 10-15% Tris-HCl, Biorad).

### 2.6.2 Secondary sample preparation

Sample lanes, containing approximately 10µg of protein, were excised from the gel and sliced horizontally into equal gel slices of approximately 2.5mm. Individual gel slices were finely chopped (~1mm$^3$), transferred to 0.5ml Eppendorf tubes and processed using standard in-gel reduction, alkylation and trypsinolysis steps. Digest supernatants of 20µl final volume were transferred to HPLC sample vials and stored at 4$^o$C until required for LC-ESI-MS/MS analysis.

### 2.6.3 HPLC

Liquid chromatography was performed using an Ultimate 3000 nano-HPLC system (Dionex) comprising a WPS-3000 well-plate micro auto sampler, a FLM-3000 flow manager and column compartment, a UVD-3000 UV detector, an LPG-3600 dual-gradient micropump and an SRD-3600 solvent rack controlled by Chromeleon chromatography software. A micropump flow rate of 246µl/min was used in combination with a cap-flow splitter cartridge, affording a $^1/_{82}$ flow split and a final

flow rate of 3µl/min through a 5cm x 200µm ID monolithic reversed phase column (Dionex-LC Packings) maintained at 50$^{o}$C. Samples of 4µl were applied to the column by direct injection. Peptides were eluted by the application of a 15 minute linear gradient from 8% to 45% solvent B (80% acetonitrile, 0.1% formic acid) and directed through a 3nl UV detector flow cell. LC was interfaced directly with a 3D high capacity ion trap mass spectrometer (Esquire HCTplus$^{TM}$, Bruker Daltonics) utilising a low-volume (50µl/min max) stainless steel nebuliser (Agilent, cat. no.G1946-20260) and ESI. MS/MS analysis was initiated on a contact closure signal triggered by Chromeleon software.

### 2.6.4 Database mining

MS/MS data was analysed using the MASCOT$^{TM}$ search engine from Matrix Science. Data was mined against the NCBInr database, with "mammalia" as a taxonomical parameter and fixed modification carbamidomethyl selected. Mass tolerances were set at 0.5Da when using MS/MS data in the MASCOT$^{TM}$ search engine. Resultant molecular weight (MOWSE) scores were inspected manually and only considered significant if a minimum of 2 peptides per protein matched and, for each peptide, a minimum of 4 straight "b" or "y" ions, or better, were recorded.

## *2.7 Protein Identification by mass spectrometry*

Proteins of interest were manually cut from the 2-D gels and submitted for peptide mass fingerprinting (PMF) to the Proteomics Facility at the Moredun Research Institute (Pentlands Science Park, Edinburgh). The 87 most distinct spots were chosen for analysis. Where a group of spots was thought to represent isoforms of the same protein, at least two spots were chosen from within the group to confirm this. The selected spots were subjected to trypsin digestion (Promega sequencing grade modified trypsin). Each sample was processed and spotted on the sample well of a MALDI sample plate. Adjacent to each sample, Pepmix 1 Standard (Bruker Daltonics) was spotted onto the plate. Analysis was then carried out on a Bruker Ultraflex II MALDI-ToF-ToF mass spectrometer (Bruker Daltonics). Data for PMF

analysis was accumulated from 10x100 shot batches. Each sample was calibrated using the adjacent standard. Data was processed using Flex analysis software (Bruker Daltonics) and compiled into mass lists.

The resultant mass list was then searched against protein databases, principally NCBInr and Swissprot, using the MASCOT™ search engine from Matrix Science (accessible at www.matrixscience.com/search_form_select.html). The MASCOT™ search engine uses mass spectrometry data to identify proteins from primary sequence databases. Essentially, the masses derived from tryptic digest are compared to masses derived from computer generated, theoretical tryptic digests of primary sequences held in the available sequence databases. Comparison of the experimental and theoretical digests identifies either a precise match or an entry with the closest homology.

Mass tolerances were set at 50ppm for PMF data and fixed modification carbamidomethyl (a modification of cysteine residues by iodoacetamide). Protein identifications were only considered reliable when the MASCOT algorithm calculated a confident identity match with a p value of less that 0.05. Cross-checking of pI and MW of the sample spot to the theoretical values was used to confirm the identity.

Tandem mass spectrometry (MS/MS) was carried out as described in section 2.6, and also on a selection of samples used for PMF. Individual peptides were fragmented further and ion fragmentation data obtained by means of Bruker Lift-cell technology. This yielded more in depth sequence information and allowed greater confidence in the identifications obtained. Mass tolerances were set at 0.5Da when using MS/MS data in the MASCOT™ search engine. Protein identifications were only considered reliable if a minimum of 2 peptides per protein matched and, for each peptide, a minimum of 4 straight “b” or “y” ions, or better, were recorded

.

## *2.8 Image analysis of 2-D gels*

This analysis was undertaken to compare the following:-.

| Infection status | Day post challenge | Number of samples analysed |
|---|---|---|
| Primary infection | 0 | 5 |
| Primary infection | 11 | 5 |
| Primary infection | 21 | 5 |
| Immune challenged | 0 | 5 |
| Immune challenged | 6 | 5 |
| Immune challenged | 10 | 5 |

These samples were from 10 month old lambs. This age group was selected because, as noted in the General Introduction, they are able to mount an effective immune response against *T.circumcincta*, and hence potential important immune effector molecules would be present. The choice of days to analyse was aimed at giving coverage over the cannulated time period following challenge, in a manageable number of time-points.

Images of all gels for analysis were obtained using a flatbed scanner (ImageMaster LabScan, Amersham) in transmittance mode at 150 dots per inch (dpi). Analysis of the 2-D gels was carried out using Dymension software **(**version 2.02 Syngene, Cambridge). Due to the multiple isoforms of many of the proteins present in lymph and blood plasma, automatic spot detection was followed by manual merging of clusters of isoforms of some proteins into a single spot. Thus these grouped spots were selected in a repeatable fashion each time.

Each spot was assigned a spot volume, normalised against the total protein volume of the whole gel. The software then performed automatic spot matching between gels and calculated the ratio of change between gels. Spots undergoing a 1.5 fold change or greater between time points, in more than one animal, were selected for further statistical analysis. General linear model (repeated measures) ANOVA testing

(Mintab®13) was applied to all spots of interest and those spots where $p<0.05$ were considered to change significantly with time.

### 2.8.1 Corrections applied to automatic spot analysis

As well as the automated analysis described above, a manual comparison was also undertaken because the former is dependent on a match being formed between two spots. If a spot does not exist in one gel then no match will be formed which could lead to important changes being overlooked. This comparison was conducted using Dymension software (Syngene).

## *2.9 Silver Staining*

Silver staining was performed on initial 2-D gels and compared with Colloidal Coomasie staining techniques. Silver staining is more sensitive than Colloidal Coomasie and so was used in a trial to assess which staining method was most appropriate for the subsequent selection of protein spots and their analysis by mass spectrometry.

The gel was incubated in 5mg DTT/l $dH_2O$ for 40 minutes, then for a further 40 minutes in 0.1% w/v $NaCO_3$ in $dH_2O$. The gel was washed twice in $dH_2O$ and then again in 3% w/v $Na_2CO_3$. 3% $Na_2CO_3$ containing 25ml formalin (37-41%v/v)/100ml was then added and the gel allowed to develop until sufficiently stained. Development was stopped by adding 20ml of 2.3M citric acid per 100ml of developer. The gel was incubated in this solution for 10 minutes before being washed in 10% v/v 2.3M citric acid in $dH_2O$ for 30 minutes.

## *2.10 Western Blotting*

Western blotting uses specific antibodies to label proteins separated by SDS-PAGE then transferred to a membrane.

Proteins were separated using either 1-D or 2D-SDS PAGE. The 2D gels used for western blotting had been previously fixed and stained for image analysis and so required some processing prior to blotting. The 2-D gel was removed from its plastic backing and immersed in 1% SDS for 30 minutes. Thereafter the gel was treated as if it were a freshly run gel.

The proteins were transferred from the gel to Immobilon-P (Millipore), PVDF transfer membrane using semi-dry blotting apparatus (Sigma-Aldrich (SU20-SDB)) and the appropriate buffers (Anode buffer 1: 0.3M Tris, Anode buffer 2: 0.025M Tris, Cathode buffer: 0.025M Tris-Glycine pH 9.4), to pass a current through the gel (150mA/1 hour), and cause migration of the proteins. Successful transfer of the proteins was confirmed by staining in Ponceau stain (0.1% in 5% acetic acid), before complete de-staining in $dH_20$.

The membrane was blocked and washed a number of time using blocking buffer**,** (TBST (100mM Tris Cl pH 7.4, 150mM NaCl, 0.1% Tween 20) and soya instant formula (5g in 100ml TBST, Cow & Gate, Trowbridge, Wiltshire)), to reduce non-specific binding. Primary and secondary antibodies were then added at the concentrations detailed in Table 2.1. The antibodies were diluted to the required concentration in blocking buffer. Each antibody was incubated for 60 minutes at room temperature, prior to 6x5 minute washes in washing buffer (TNTT (10mM Tris, 0.5M NaCl, 0.05% Tween 20, 0.01% Thiourosal, pH 7.4)). Following the final wash after peroxidise labelled secondary antibody application, detection was carried out using DAB (3' 3' Diaminobenzidine, Sigma). When more sensitivity was required, proteins were visualised using a chemiluminescent peroxidase substrate (Sigma). Blots were visualised and imaged using a Kodak Digital Science Image Station 440CF imager.

**Table 2.1 Antibodies used for Western Blots**

| Target protein | Primary Antibody | | | Secondary Antibody | | | Detection System |
|---|---|---|---|---|---|---|---|
| | Description | Manufacturer | Concentration | Description | Manufacturer | Concentration | |
| Gelsolin | Mouse monoclonal [GS-2C4] to Gelsolin (Ascites) | Abcam | 1 in 1000 | Goat anti-mouse IgG (H+L) HRP conjugate | Jackson Immunoresearch | 1 in 5000 | Chemiluminescent Substrate (Sigma) |
| Claudin 1 & 3 | Rabbit anti-claudin 1 (250µg/ml) | Zymed | 1 in 250 | Donkey F(ab')2 anti-rabbit HRPO (200µg/ml) | Jackson Immunoresearch | 1 in 1000 | Chemiluminescent Substrate (Sigma) |
| Occludin C terminus | Rabbit anti-occludin (250µg/ml) | Zymed | 1 in 250 | Donkey F(ab')2 anti-rabbit HRPO (200µg/ml) | Jackson Immunoresearch | 1 in 1000 | Chemiluminescent Substrate (Sigma) |
| ZO-1 | Rabbit anti-ZO-1 (250µg/ml) | Zymed | 1 in 250 | Donkey F(ab')2 anti-rabbit HRPO (200µg/ml) | Jackson Immunoresearch | 1 in 1000 | Chemiluminescent Substrate (Sigma) |
| Occludin N terminus | Rabbit anti-occludin (N-term) (250µg/ml) | Zymed | 1 in 250 | Donkey F(ab')2 anti-rabbit HRPO (200µg/ml) | Jackson Immunoresearch | 1 in 1000 | Chemiluminescent Substrate (Sigma) |

### 2.10.1 Urea extraction of abomasal proteins for subsequent Western Blotting

This protocol was described by Pemberton et al. (Pemberton *et al.* 2004b). Protein was extracted from abomasal tissue for subsequent probing with antibodies to the tight junction proteins.

500µl of urea extraction buffer (8M urea (Sigma ultrapure), 2% CHAPS (Sigma ultrapure), 0.4% DTT (Sigma ultrapure), 10mM Tris-HCl, pH 7.5, one protease inhibitor tablet (complete-mini, EDTA-free, (Roche) per 7 ml of buffer) was added to 50mg of tissue sample in a Q BioGene Green capped tube with Lysing Matrix D. The contents were then homogenised for 40 seconds before being placed on ice for 5 minutes. The tube was then returned to the homogeniser for a further 40 seconds and then immediately incubated on ice for 10 minutes. The liquid contents of the tube were transferred to a QIAshredder spin column (Qiagen). The flow through from the tube was collected as this contains the extracted protein. Proteins were then separated using reducing SDS-PAGE with the gel being run at 100V for 80 minutes, the lower voltage being used to minimise local heating and possible gel distortion due to the presence of the urea. Western blotting was carried out as per the method detailed above.

## *2.11 Acute Phase Protein Assays*

### 2.11.1 Serum Amyloid A assay

The blood and lymph samples were analysed for serum amyloid A content using the Tridelta Phase$^{TM}$ range SAA kit, which is a solid phase sandwich Enzyme Linked Immunosorbant Assay (ELISA).

All blood plasma samples were diluted 1 in 500 prior to assay, and lymph diluted 1 in 300 prior to assay, in the dilution buffer supplied in the kit. Calibrators were supplied and diluted as per manufacturers instructions (again in dilution buffer) to give 6 standards equivalent to 300, 150, 75, 37.5, 18.8 and 0 ng/ml SAA (Bovine).

These were used to establish a standard curve from which the concentration of unknowns was derived. 50µl of each diluted sample or calibrator, in duplicate, was added to the ELISA plate supplied (coated in monoclonal anti-SAA antibody). The plate was incubated at 37$^{o}$C for 60 minutes. The plate was then washed four times in wash buffer ((Phosphate buffered saline –Tween 20) - supplied with kit). 100µl of biotinylated anti-SAA monoclonal antibody was then added to each well, and the wells mixed gently by tapping the sides. The plate was incubated in the dark for 60 minutes. The plate was then washed four times in wash buffer and tapped dry. 100µl of Streptavidin-Horse Radish Peroxidase conjugate was added to each well, and the plate incubated in the dark for a further 30 minutes at room temperature. Following a further four washes in wash buffer, 100µl of TMB (tetramethylbenzidine) substrate was added and the plate incubated for a further 30 minutes in the dark. The stop solution (50µl) provided was then added to each well before the absorbance was measured at 450nm.

### 2.11.2 Haptoglobin assay

The blood and lymph samples were analysed for haptoglobin content using the method described by Eckersall et al (Eckersall *et al*. 1999). This work was carried out at Glasgow University Veterinary School, with the aid of David Eckersall and Mary Waterston. Briefly, haptoglobin concentrations were measured by an automated biochemical assay. The assay was based on the capacity for haptoglobin (Hp) to bind to haemoglobin (Hb), and the resulting innate peroxidase activity of the Hp-Hb complex. This particular assay uses the reagent SB-7, which is a solution of peroxidase chromogen, detergent and protein binding inhibitors in a citrate buffer. This eliminates the effects of albumin which could otherwise cause falsely high results due to its effects on the peroxidase reaction (Eckersall *et al.* 1999). In the presence of the substrate (hydrogen peroxide), the peroxidase activity causes a colour change in the chromogen SB-7 and absorbance is measured at 600nm. Samples of known haptoglobin concentration were used to calibrate the assay and create a standard curve from which the haptoglobin concentrations of the test samples were calculated.

### 2.11.3 Alpha-1 acid gycoprotein assay

The alpha-1 acid glycoprotein content of the blood and lymph samples of interest was analysed using a commercially available Ovine alpha-1 acid glycoprotein measurement kit (supplied by J-Path Inc., Tokyo, Japan). The assay is based on immunodiffusion and was conducted exactly as outlined in the kit instructions. As the sample diffuses radially from the well, the alpha-1 acid glycoprotein present in the sample forms a precipitate as it comes into contact with the specific antiserum to ovine α-1 AGP. This forms a visible ring, the diameter of which is directly proportional to the concentration of AGP in the sample. The sample concentration is determined by plotting the diameter of the test sample ring on the reference curve created by the two standards of known concentration provided in the kit.

## *2.12 Immunohistochemistry techniques*

Abomasal fold sections used for immunohistochemistry to study tight junction proteins, were fixed in paraformaldehyde (4% in PBS) and then paraffin embedded. The collection, fixation and cutting of tissue sections was performed by Ms Judith Pate and paraffin embedding was carried out by Pathology services at The University of Edinburgh, Easter Bush Veterinary Centre.

Sections were dewaxed by successive immersion for 2mins each in Xylene I; Xylene II; Ethanol I; Ethanol II; 95% ethanol; 90% ethanol; 70% ethanol and, finally, distilled water.

### 2.12.1 Antigen Retrieval

Following formalin fixation, antigen retrieval methodology aids the display of antigens by breaking protein cross-links formed during the fixation process, thereby revealing hidden antigenic sites.

Sections were heated for 30 minutes in 1 litre of antigen retrieval buffer (10mM Tris, 1mM EDTA, 0.05% v/v Tween 80, pH9.0). Heating was carried out in a pressure

cooker placed in an 800W microwave oven set at high power. The sections were heated for 10 minutes prior to washing in distilled water.

### 2.12.2 Blocking slides

Non-specific antigenic binding sites were blocked prior to application of antibody in order to reduce non-specific staining. The sections were placed in PBS pH7.4, 0.05% v/v Tween 80 prior to loading into a Sequenza apparatus (Thermo Electron, Germany). PBS was used to load the slides and the reservoirs were also filled with PBS following successful loading of the slide into apparatus. This PBS was allowed to drain through and hence wash the slide. A further wash was carried out using the PBS/Tween 80 buffer. 100µl of blocking buffer (500µl of normal sheep serum in 5ml PBS/Tween 80 buffer) was then added to each slide and allowed to drain through.

### 2.12.3 Staining procedure

Details of antibodies used can be found in Table 2.2. 100µl of primary antibody diluted in blocking buffer, was added to each slide as appropriate. The slides were then incubated in primary antibody for 60 minutes at room temperature. Following two washes in PBS, 100µl of secondary antibody (fluorescently labelled conjugate) was added to each slide, incubated for 30 minutes at room temperature, in the dark, after which the slides were again washed with PBS. 100µl of sybr green (Invitrogen), diluted 1:1000 in PBS/Tween 80 buffer, was then added and this was incubated for 15 minutes, again in the dark. (The sybr green was included as a DNA binding counterstain). The slides were than washed for a final time in PBS prior to mounting in 60µl MOWIOL, a high quality, anti-fade medium (Hoechst). For every antibody there was also a negative control slide. This slide was incubated with rabbit IgG isotype control (IgG extracted from control rabbits) (Jackson Immunoresearch), and then treated as per the other slides.

### 2.12.4 Visualising and Imaging Slides

Every slide was individually examined and digital images stored using the Zeiss Axiovert 200M microscope (Carl Zeiss, Germany). Rhodamine Red X (RRX) excitatory wavelength is 570nm, with an emission peak at 590nm. Indodicarbocyanine 5 (Cy5) excitatory wavelength is 650nm, with an emission peak at 670nm. Images were captured with an Axiocam MRm digital camera and then processed using Axiovision software, release 4.7.2. Images shown in this thesis used x40 objective lens.

**Table 2.2 Antibodies used for immunohistochemistry in the study of tight junction proteins**.

| **Target protein** | **Primary Antibody** | | | **Secondary Antibody** | | | **Fluorescent Label** |
|---|---|---|---|---|---|---|---|
| | Description | Manufacturer | Conc. | Description | Manufacturer | Conc. | |
| Claudin 1 & 3 | Rabbit anti-claudin 1 | Zymed | 2µg/ml | Donkey F(ab')2 anti-rabbit RRX | Jackson Immunoresearch | 2µg/ml | RRX |
| Occludin C terminus | Rabbit anti-occludin | Zymed | 1µg/ml | Donkey F(ab')2 anti-rabbit RRX | Jackson Immunoresearch | 2µg/ml | RRX |
| ZO-1 | Rabbit anti-ZO-1 | Zymed | 2µg/ml | Goat F(ab')2 anti-rabbit Cy5 | Jackson Immunoresearch | 2µg/ml | Cy 5 |
| Occludin N terminus | Rabbit anti-occludin (N term) | Zymed | 1µg/ml | Donkey F(ab')2 anti-rabbit RRX | Jackson Immunoresearch | 2µg/ml | RRX |

## *2.13 Polymerase Chain reaction (PCR)*

### 2.13.1 RNA extraction and purification from tissues

RNA was extracted from tissues, which had been stored at –20$^{o}$C in RNAlater$^{TM}$ RNA Stabilisation Reagent for tissues (Qiagen). Extraction was carried out using FASTPREP/RNeasy (Qiagen) kits according to the instructions. RNA was stored at –80$^{o}$C until required. RNA concentration was determined on the basis that 1 unit absorbance at 260nm is equivalent to 44µg of RNA per ml. RNA purity was evaluated using OD260:OD280 with a value greater than 1.5 considered acceptable.

### 2.13.2 cDNA synthesis

The RNA, extracted as above, was diluted to a final concentration of 0.1µg/µl and 10 µl (ie.1 µg RNA ) was then used in the subsequent reverse transcription reaction. This was conducted using the ImProm-II ™ Reverse Transcription System (Promega) [A3800] according to the manufacturer's instructions. First strand synthesis was initiated by Oligo(dT)$_{15}$ priming.

Each reverse transcription reaction (cDNA sample) was then diluted 5 fold and stored at –20$^{o}$C.

### 2.13.3 PCR

The procedure of primer directed enzymatic amplification of DNA with a thermostable polymerase was first described by Saiki et al 1988 (Saiki *et al.* 1988). In brief, the reaction involves three steps. Firstly, an initial denaturation step, whereby the template cDNA and the primers are heated to 95$^{o}$C. This causes disruption of hydrogen bonds between complementary strands of DNA to yield single stranded DNA.

Following this there is a period of lower temperature (50-65$^{o}$C, dependent upon the calculated melting temperature of the primers). During this time the primers align with and form hydrogen bonds with the complementary section of template DNA.

This is called the annealing step. The enzyme, DNA Polymerase then binds to the double stranded regions (primer-template hybrid) and begins DNA synthesis.

The final step in PCR is the extension/elongation step. The sample is now heated to the optimum temperature for activity of the DNA polymerase (usually around 70-80$^{o}$C). This enzyme induces the formation of a complementary strand of DNA to the template strand, by laying down nucleotides (dNTPs) in a 5' to 3' direction. If there are no limiting factors, the amount of DNA target is doubled at each elongation step, leading to exponential amplification of the target DNA. These steps are repeated a number of times depending on how much target DNA is desired, and indeed how much template DNA was present to begin with.

### 2.13.4 Primer Design

Available sequences of the gene(s) of interest were extracted from the NCBI database (http://www.ncbi.nlm.nih.gov/) with ovine sequence selected when available. On these occasions, Primer 3 online primer design tool (http://frodo.wi.mit.edu/) was used to select PCR primers. However, in most cases, there was either no or incomplete ovine sequence available. In these instances, the sequence of the gene of interest in at least three other species (principally *Homo sapiens*, *Mus muscularis* and *Bos taurus*), were used and aligned using the Clustal W alignment tool available online (http://www.ebi.ac.uk/Tools/clustalw2/index.html). From this alignment profile, the areas showing maximum interspecies homology were selected from which to design primers for ovine sequence detection. All primers were supplied by MWG-Biotech. Primer details are given in the Table 2.3.

**Table 2.3 Primers used for PCR (excluding real-time PCR)**

| Gene of interest | Origin of Sequence | Forward primer (5'-3') | Tm (°C) | Reverse primer (5'-3') | Tm (°C) | Product Size (bp) | Annealing temp. (°C) | Cycle no. |
|---|---|---|---|---|---|---|---|---|
| Sheep ATPase | *Ovis aries* ATPase, Na+/K+ transporting, α-1 ploypeptide NM01009360 | GCT GAC TTG GTC ATC TGC | 56.0 | CAG GTA GGT TTG AGG GGA TAC | 59.8 | 167 | 60 | 31 |
| SAA3 | *Bos taurus* serum amyloid A3, mRNA BC108181 | GCC AGA GAT GGG GGA CAT TC | 61.4 | TCC CTG CCC TTA GGG ACT CA | 61.4 | 396 | 61 | 35 |
| AGP | *Bos Taurus* mRNA for α-1 acid glycoprotein (AGP gene) AM403243.2 | TGC CAC CAT GGA CTT GCT CT | 59.4 | GAA GGA CAC CCC CAC GTT CT | 61.4 | 334 | 61 | 31 |
| Haptoglobin | *Bos Taurus* haptoglobin (Hp), mRNA NM001040470.1 | ATG TGG GGA AGA ACC AGC TT | 57.2 | AAG GTA GGC AGA TGG GCA TT | 57.4 | 135 | 57 | 34 |
| Gelsolin (cytoplasmic variant) | Composite sequence from available ovine EST database | CCG CAG GTG GTA CTG GGA AC | 63.5 | CCT CAG CTG CAC CGT CTT CA | 61.4 | 263 and 335 | 62 | 38 |
| Geslolin (plasma variant) | Composite sequence from available ovine EST database | GCA GGG GCG AGT GAC C | 59.4 | AGG TCG AAC TTC TCC ACA CG | 59.4 | 114 | 58 | 40 |
| pGEM®-T plasmid | Promega T7 and SP6 | TAA TAC GAC TCA CTA TAG GG | 53.2 | TAT TTA GGT GAC ACT ATA G | 48.0 | * | 48 | 30 |

* variable, depending on length of insert sequence

### 2.13.5 PCR conditions

For each gene of interest the number of PCR cycles and annealing temperature differed. However, the general conditions are given below and annealing temperatures for individual genes are given in Table 2.3 above. In general terms, PCR reactions comprised 10X Buffer (5µl); 2.5 unit PCR Taq polymerase (Roche Diagnostics GmbH, Mannheim, Germany), 5 µl of a 2mM dNTP mix; 5 µl each of 2µM stock solutions of the forward and reverse primers mixed with 0.5 µg template cDNA. Following an initial denaturation step (94$^{o}$C for 2 minutes), x amplification cycles (numbers defined and annealing temperature used for each target are given in Table 2.3) were conducted. These were 94$^{o}$C for 30 seconds (Denaturation step), annealing temperature for 30 seconds (Annealing step), 72$^{o}$C for 10 minutes (Elongation step) with a final elongation step of 72$^{o}$C for 10 minutes.

For every PCR run carried out, a negative control was included for each gene of interest (no template DNA). The reaction products were fractionated and visualised using agarose gel electrophoresis.

### 2.13.6 Agarose gel electrophoresis.

Agarose (2% final concentration) was melted in 1X TAE buffer (Tris acetate 40mM, 1mM EDTA) with GelRed (Biotium Inc) added as per manufacturer's instructions. The mixture was poured into a plastic casting tray containing a plastic comb to create sample wells. The gels were allowed to cool and solidify prior to loading of the sample. Samples were mixed 1:5 with 6X Blue/Orange loading dye (Promega), loaded into wells and ran at 100V until the dye front had reached the end of the gel.

### 2.13.7 Semi-quantitative PCR

Semi-quantitative PCR relies upon stopping the PCR reaction during the exponential phase (before any of the reaction components become limiting). The resultant product was then run on an agarose gel, stained with Ethidium Bromide and imaged using the Kodak Digital Science Image Station 440CF imager. The image was then subjected to densitometry analysis, to establish the timing of the exponential phase of

the reaction, using Image J software, available at http://rsbweb.nih.gov/ij/. Subsequent PCRs on samples of interest were run at the optimum number of cycles for semi-quantitative analysis.

### 2.13.8 Real-time PCR (quantitative PCR; qPCR)

This technique was used to quantify, based on transcript abundance, changes in gene expression during infection. The real-time assays were conducted using TaqMan® probe technology (Applied Biosystems). The TaqMan® probe consists of two dyes on a single-stranded DNA fragment which is complementary to a region of template DNA which lies between the forward and reverse primer i.e. within the region to be amplified. The quencher dye (TAMRA™) prevents the reporter dye (FAM™) from fluorescing when excited by the laser on the Thermo-cycler (ABI Prism 7500), as long as the two dyes remain within close proximity to each other. During the elongation step of PCR, the reporter dye (at the 5' end) is separated from the fragment and in doing so, gains the ability to fluoresce. By measuring fluorescence at the end of the elongation step, a measure of the amount of template DNA is gained. The opportunity for fluorescence hence increases as the DNA copy number increases.

Primers and probes for real-time assays were designed using Primer Express® Version 2.0 software (Appiled Biosystems) (Table 2.4). TaqMan qPCR was carried out in a total volume of 25 µl containing 0.1µg template DNA (or water in the case of no-template controls), and 12.5µl Platinum® Quantitative PCR Supermix UDG (Invitrogen). The optimised primer and probe concentrations for each assay are shown in Table 2.5

Reactions were amplified using an ABI Prism 7500 Thermo-cycler (Applied Biosystems) under the ABI Prism 7500 software using cycling conditions of 50$^{o}$C for 2 min, 95$^{o}$C for 2 min, followed by 40 cycles of 15 sec at 95$^{o}$C and 1min at 58$^{o}$C (57$^{o}$C for Haptoglobin).

For these experiments, the endogenous control gene was sheep ATPase, and the calibrator sample was uninfected abomasum.

**Table 2.4 Primers used in quantitative PCR assays**

| Target gene | Source sequence | Forward primer sequence | Tm (°C) | Reverse primer sequence | Tm(°C) | Probe sequence | Tm(°C) | Amplicon Length (bp) |
|---|---|---|---|---|---|---|---|---|
| SheepATPase | *Ovis aries* ATPase, Na+/K+ transporting, α-1 ploypeptide NM01009360 | AAC GGC TTC CTC CCT AAT CAC | 58 | GTC GTT GAT CCA ACG GTC ATC | 59 | TGC TGG GCA TCC GAG TGA CCTG | 68 | 66 |
| SAA3 | *Bos taurus* serum amyloid A3, mRNA BC108181 | CCA ACT ACA GGG GTG CAG AC | 57 | GAA TCC TCC CGT ACC TGG TC | 57 | ACG CCC GCG GAA ACT ATG ACG | 67 | 174 |
| AGP | Ovine sequence obtained from sequencing rtPCR product | GCT TTC CGA AAC CCT GAG TAC A | 59 | GCC TGG GCT CAA AGT AAA AGA A | 59 | AGT CGG CTA GAG CAA TCC AGG CGG | 69 | 73 |
| Haptoglobin | Ovine sequence obtained from sequencing rtPCR product | ATG TGG GGA AGA ACC AGC TT | 57 | AAG GTA GGC AGA TGG GCA TT | 57 | CTC CAC CCT GAC CAC TCC AAG GTA GAC | 67 | 134 |
| Gelsolin | Ovine sequence constructed from Ensembl Genome Browser (Figure 5.6) | GGC AGG CAG GCC AAC AC | 60 | GGT AGT CCA TCT TGG AGA TGA AGT C | 58 | AGG AAG GCC GCC CTC AAA ACA GC | 69 | 76 |

**Table 2.5 Optimised real-time PCR assay components**

| Target gene | Optimised assay components | | | | Reaction Efficiency (%) |
|---|---|---|---|---|---|
| | Annealing temp. (˚C) | F primer [ ] (nM) | R primer [ ] (nM) | Probe [ ] (nM) | |
| Sheep ATPase | 58 | 50 | 900 | 200 | 104 |
| SAA | 58 | 900 | 900 | 225 | 100 |
| Haptoglobin | 57 | 900 | 900 | 100 | 105 |
| AGP | 58 | 900 | 900 | 200 | 105 |
| Gelsolin | 58 | 300 | 900 | 200 | 93 |

### 2.13.9 Real-time PCR data analysis

Data generated by real-time PCR was analysed by means of the Comparative Ct method (ΔΔCt). This relies upon the target gene having a similar reaction efficiency to the endogenous control which, in this case, was in the region of 100%. This method negates the need for standard curves and gives a relative quantity of the gene mRNA in relation to a calibrator gene. The crossing threshold (Ct) of an assay is the number of cycles required for the fluorescent signal to cross a pre-determined threshold (either set manually or automatically by the software). This threshold is selected to occur at the beginning of exponential amplification, once the fluorescent signal exceeds that of the background level. ΔCt is first calculated by subtracting the Ct value of the target gene (mean of four experimental replicates), from that of the endogenous control (also the mean of four experimental replicates) for that particular mRNA sample. The ΔΔCt value is calculated by subtracting the ΔCt value of the target gene from the ΔCt of the calibrator sample. Using the ABI Prism 7500 system (Applied Biosystems), this is known as an RQ (Relative Quantification) study. RQ study plate must be selected on software prior to running the PCR. This type of study does not yield actual copy number data. This information is from the Applied Biosystems' literature – Relative Quantitation using comparative Ct Getting Started Guide ( docs.**appliedbiosystems**.com/pebiodocs/04347824.pdf).

A t-test was carried out for each pair wise comparison (ΔCt calibrator versus ΔCt target gene). This was a two-sample, unpaired, two-sided t-test, where variances were not assumed equal (greater variation occurred in the liver samples as demonstrated by dot plotting the data). Significance level was set at 5%. The total number of tests carried out was 72. The significance level then had to be adjusted for the false discovery rate due to multiple tests. The multiple testing correction used was Benjamini and Hochberg False Discovery Rate correction (Benjamini & Hochberg 1995).

## *2.14 Other Molecular Biology procedures*

### 2.14.1 Sequencing from purified PCR product

Purification of PCR product for sequencing was carried out using the High Pure PCR Product Purification Kit (Roche, 11732668001). The products from four identical 50µl PCR reactions were combined and purified for each sequencing reaction (total volume 200µl). The purification procedure was carried out as described in the instructions accompanying the kit. DNA sequencing was conducted by MWG-Biotech.

### 2.14.2 Sequencing from plasmid DNA following cloning

#### 2.14.2.1 Extraction and purification of DNA from PCR product

The DNA sequence used as the insert was obtained either from purified PCR product (QIAquick® PCR Purification Kit, Qiagen) or from DNA extracted and purified from gel slices excised from Gel Red™ (Biotium Inc) stained agarose gel of PCR product. This was done using the QIAquick® Gel Extraction Kit (Qiagen).

The QIAquick® spin column technology is based on a silica membrane which, in the presence of high salt concentrations, absorbs DNA while allowing contaminants to pass through the column during the wash stages. Elution of the bound DNA, following removal of contaminants by the various wash/spin cycles, is most efficient under basic conditions and in a low salt concentration. Hence the DNA was eluted using either 30µl of water or Buffer EB (10mM Tris-HCl, pH 8.5).

Both the QIAquick® PCR Purification Kit and the QIAquick® Gel Extraction Kit were used according to the manufacturer's instructions.

#### 2.14.2.2 Ligation reactions

DNA sequence of interest was inserted into the pGEM®-T Vector System (Promega) as described by the manufacturer. Ligation was catalysed using T4 DNA ligase.

#### 2.14.2.3 Preparing LB/Ampicillin/IPTG/X-Gal plates

LB Agar (10g Bacto®-Tryptone, 5g yeast extract, 5g agar in 1 litre $dH_2O$, autoclaved and stored at room temperature) was melted in a microwave and then equilibrated to 50$^{o}$C in a water bath. 5mg of ampicillin, 250µl (100mM stock) IPTG and 80µl of X-Gal were added per 50ml of agar. The agar was decanted into the required number of plates. After setting at room temperature, plates were stored in the dark until use, and refrigerated if not for use within 12 hours of preparation.

#### 2.14.2.4 Transformation reaction

An aliquot of the ligation reaction (2µl) added to 50µl of JM109 High Efficiency Competent Cells (Stratagene) and this mixture was then incubated on ice for 20 minutes. The cells were then heat-shocked in order to open up the cell walls in preparation for plasmid entry, by placing the tube in a 42$^{o}$C water bath for 45-50 seconds. The tube was then immediately returned to ice for 2 minutes. 500µl of SOC Medium was then added before incubation at 37$^{o}$C for 1.5 hours with shaking.

100µl of each transformation culture was then added to each duplicate LB/ampicllin/IPTG/X-Gal plate. The plates were incubated overnight (16-24 hours) at 37$^{o}$C. Prior to colony selection, the plates were refrigerated (4$^{o}$C) in order to enhance development of blue colour where present.

#### 2.14.2.5 Cloning

Usually, six white colonies were selected and each added to 50µl water, for cloning and colony PCR, and grown overnight with constant shaking at 200 rpm at 37$^{0}$C in L-broth containing 10µl of Ampicillin (250mg/ml).

#### 2.14.2.6 Colony PCR

This was conducted with standard PCR conditions using T7 and SP6 primers which are specific for the plasmid sequence flanking the insertion sequence and 10µl of bacterial colony diluted in water from above as target.

#### 2.14.2.7 Extraction and purification of plasmid DNA

Plasmid DNA was purified from overnight LBroth/Ampicillin cultures using the Wizard® Plus SV Minipreps DNA Purification System (Promega) according to the kit instructions. The plasmid DNA concentration was determined using the Nanodrop® and the DNA sample diluted to the appropriate concentration required for sequencing. This was conducted by MWG-Biotech.

### 2.14.3 DNA sequencing

DNA sequencing was carried out by MWG Biotech using the cycle sequencing technology (dideoxy chain termination/cycle sequencing) on ABI 3730XC sequencing machines.

Both forward and reverse sequence data were always requested. In this way any ambiguities in the sequence could be overcome by comparing the sequences, in combination with studying the chromatogram files (using Chromas Lite 2.0 software) to determine the correct sequence.

# 3 Chapter 3: The proteomic analysis of abomasal lymph

## *Summary*

In an effort to detect potential local changes occurring in the abomasum during *T.circumcincta* infection, lymph draining the abomasum was collected and analysed. Lymph analysis has the advantage over analysis of blood in that locally produced proteins should be at a higher concentration in lymph, and hence change should be more distinct. It was hoped that this would yield further information regarding potential novel effector proteins involved in immunity to the disease, or indeed proteins involved in the pathology of the condition. Lymph protein kinetics were monitored in both naive animals undergoing a primary challenge and also in immune animals subjected to a secondary challenge. The change in protein levels over time, or indeed the presence or absence of a particular protein in one challenge model and not the other, would highlight this candidate for further investigation as a potential molecule of interest.

Lymph duct cannulation allows the collection of lymph draining from a defined anatomical region. Proteomic analysis of that lymph offers a potentially valuable insight into the immuno-inflammatory response of that particular region. In this study, ovine efferent gastric lymph was used to monitor the proteomic changes occurring in the tissue fluid of the abomasum, in response to infection with the parasitic nematode, *Teladorsagia circumcincta*. Lymph, collected daily for up to 3 weeks post-infection, was analysed by means of 2-DE and subsequent gel analysis using densitometry software. In addition, the composition of the lymphatic proteome was further explored by means of MALDI-TOF and MS/MS analyses. The concentrations of gelsolin, α-1 B glycoprotein and hemopexin were altered significantly ($p<0.05$) following infection.

## 3.1 Introduction

A greater understanding of the local mechanisms involved in the pathology caused by *Teladorsagia circumcincta*, and in immunity to the parasite, is necessary if immunological control is to become an achievable goal.

Much of the present understanding of the immune response to *T. circumcincta* has derived from analysis of lymph obtained from cannulation of the efferent common gastric lymph duct (Smith *et al.* 1983a; Smith *et al.* 1984). The abomasal mucosa of a worm free sheep is immunologically quiescent, with minimal evidence of any inflammatory or immunological stimulus. Thus, afferent lymph draining from the interstitial tissues to the gastric node, and the lymph efferent from the node, reflects this relatively quiescent status of the mucosal tissues. Efferent gastric lymph from worm free sheep contains predominantly small lymphocytes (Smith *et al.* 1983b), a cellular composition which is quite different from that of intestinal lymph (Adams & Cripps 1977). Intestinal lymph has a much higher background immunological activity.

Most lymph proteins are derived from blood. They enter the lymph by means of diffusion, filtration and vesicular transport and hence lymph contains, predominantly, all of the same proteins as blood, albeit at a lower concentration (Interewicz *et al.* 2004). In addition it contains proteins produced locally by the surrounding tissues and also antigens which gain access through the mucosal surfaces. The local proteins and antigens enter lymph from the interstitial fluid via lymphatics afferent to the node and, in the case of efferent lymph, from the lymph node itself. That lymph is more than a filtrate of blood was shown by Leak et al. (Leak *et al.* 2004) who were able to demonstrate the presence of protein spots separated by 2-DE in lymph which were absent from blood. By studying lymph the likelihood of observing these proteins of local origin should be enhanced, in comparison to looking at the systemic circulation where any local contribution will be massively diluted and hence likely to be difficult to observe. A key advantage of using lymph is to monitor changes over

time in the same sheep. This is potentially more powerful than the single time point samples that can be obtained when killing sheep and sampling gastric folds.

In this study, the proteome of ovine gastric efferent lymph was investigated during the course of infection with the gastric nematode, *T. circumcincta.* The specific aim was to monitor changes to the lymph proteome associated with the immunoinflammatory response occurring in the mucosa and in the gastric lymph node from which the lymph was collected.

It was hypothesized that proteomic analysis of this lymph would yield further information regarding the pathology occurring locally within the abomasum and possibly help identify potential effector molecules involved in immunity to infection with *T.circumcincta*. The investigation of animals of different immune status would help to highlight proteins of interest, as would changes in the kinetics of these proteins over the time course of an experimental infection period. A thorough search of the appropriate literature suggests this is the first time that disease progression has been monitored by proteomic analysis of lymph.

Analysing such a complex proteome as blood or lymph plasma presents some technical challenges. The 10-15 most abundant proteins (present in mg/ml quantities) constitute less that 0.1% of the total number of proteins, whilst accounting for greater than 95% of the total protein mass of blood and lymph (Anderson & Anderson 1998). The total protein loading which can be separated by 2-DE is limited. Therefore, the less abundant proteins may be below the detection limit of the technique. However, 2-DE remains a useful "global" technique to screen complex proteomes in order to identify particular target proteins of interest which can then be followed up by alternative, more sensitive methods.

The problems encountered in analyzing mammalian plasmas have been highlighted by a recent worldwide study aiming to further define the human plasma proteome (HUPO Plasma Proteome Project) (Omenn *et al.* 2005) and identify possible biomarkers. This collaborative study looked at various methods of teasing apart the components of the mixture, including by the pre-fractionation of plasma prior to

individual protein separation by 2-DE (Barnea *et al.* 2005; Echan *et al.* 2005; Pieper *et al.* 2003). The two most abundant proteins in blood (and lymph) are albumin and IgG, and hence removal of these will effectively increase the total loading of remaining proteins possible on a 2D gel. From previous experience in the laboratory, and from various publications (Echan *et al.* 2005; Fountoulakis *et al.* 2004; Fu *et al.* 2007), including those of the HUPO, it is known that Protein G Sepahrose (Sigma-Aldrich) is successful in removing the majority of IgG from blood plasma and so in this study we adopted this method and trialled its ability to carry out a similar function on lymph. Various groups in the HUPO consortium also looked at the use of immunodepletion, using antibodies directed against specific plasma components. Unfortunately, as the target species of this study is *Ovis aries*, fewer reagents are commercially available for such immunodepletion procedures. However, a commercial IgY spin column (IgY protein partitioning kit; Beckman Coulter) effective in removing albumin from bovine samples, despite the IgY antibodies being of the anti-human form, was available. Given the relative success demonstrated by the use of IgY immunodepletion in the HUPO study (Hinerfeld *et al.* 2004; Huang *et al.* 2005), this column was used in an attempt to deplete ovine lymph of the majority of albumin. Huang et al. (Huang *et al.* 2005) found that the use of chicken antibody (IgY) as opposed to mammalian IgG was advantageous as the Fc region of the IgY antibody did not bind complementary mammalian proteins such as complement, rheumatoid factors and IgM and hence the depletion was rendered more specific. The removal of albumin and IgG by these methods would allow relatively greater loading of the less abundant proteins, making it more likely that they would be detected and allowing their kinetics with infection to be monitored.

## *3.2 Results - Depletion of lymph*

In order to permit greater loading of less abundant proteins, all lymph and plasma samples were depleted of albumin and IgG prior to running. Loadings of depleted lymph, equivalent to 80 µl of whole lymph, were applied to each 18cm IPG strip

(Figure 3.1(B)). In contrast, loading 4 µl of whole lymph caused overloading artefacts with poor focussing and minor spots being masked by large streaks of IgG and albumin (Figure 3.1 (A)). Following depletion, transferrin became the protein of greatest abundance and hence the factor restricting the amount of total protein which can be successfully separated on the gel. Protein spots previously masked by albumin and IgG, such as hemopexin and fibrinogen, became evident.

The method employed for IgG depletion proved effective (Figure 3.2(A)). The protein G binding was also found to be quite specific as shown by 2-DE of the elution fraction from the binding column (Figure 3.2(B)). Albumin removal was generally successful (Figure 3.2(C)), but residual albumin was detectable to varying degrees in all samples (Figure 3.6). Albumin depletion appeared to be less specific than that of IgG (Figure 3.2(D)), because additional non-albumin spots were present in the elution fraction from the column.

In order to further assess the inadvertent removal of other, potentially interesting proteins, by the depletion methods, further analysis of the elution fractions from the columns was carried out, using lymph from an uninfected animal. For each method, the fraction which bound to the column was eluted and separated by means of 1-D SDS PAGE. The resultant lane was then divided into 18-22 horizontal sections, and each section analysed by means of LC-ESI-MS/MS. The data obtained is presented in Table 3.1.

Other than IgG and the contaminants keratin and trypsin, only 2 other proteins were identified as having been inadvertently removed during IgG depletion – complement C4 and peptides homologous to human Ran binding protein 2.

LC-ESI-MS/MS analysis of the proteins bound to the albumin column revealed that most of these additional spots present on the 2-D gel (Figure 3.2(D)) were actually albumin fragments and isoforms. Albumin was present in 15 of 18 gel slices analysed by LC-ESI-MS/MS. Only two additional proteins were identified as having been inadvertently removed by the anti-BSA IgY column – histone H4 and transthyretin precursor (Table 3.1).

**Figure 3.1: Comparison of whole lymph and depleted lymph on 2D gels.**

**Gel A shows 2D separation of whole lymph (4µl) prior to depletion. Gel B shows lymph (80µl of whole lymph) following depletion of IgG and Albumin. Selected proteins have been labelled (as identified by MALDI-TOF).**

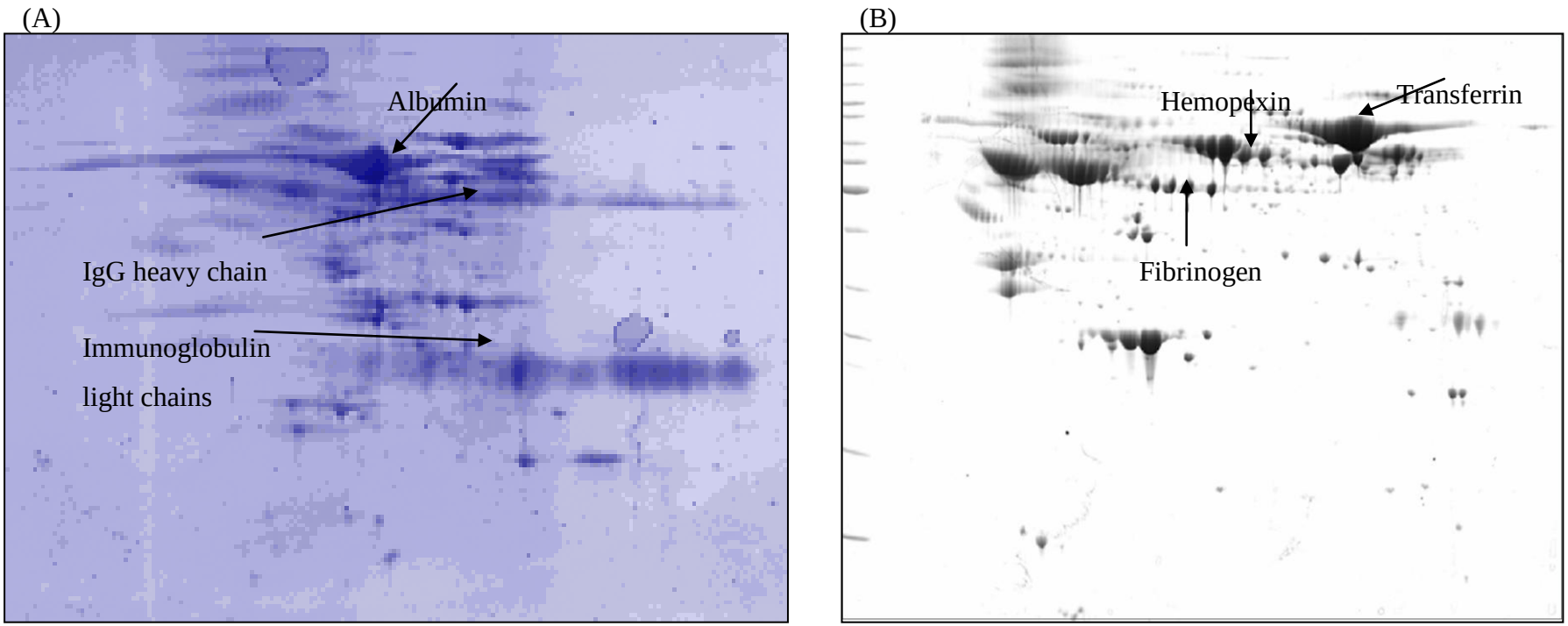


**Figure 3.2. Depletion of the two most abundant proteins from lymph. 1-D SDS PAGE gel (A) showing whole lymph (WL) and lymph depleted of IgG (DL) using Protein G (see Materials & Methods). The gel was run under non-reducing conditions and the arrow indicates IgG. The elution fraction from the Protein G column was separated by 2-DE (B), and shows multiple isoforms of Ig heavy and light chains, with little evidence that any additional proteins were removed by the column. Non-reducing 1-D SDS PAGE gel showing albumin depletion (C). Whole lymph (WL) is shown in the left lane and depleted lymph (DL) in the right. The depletion of the albumin band is indicated (arrow). The elution fraction from the albumin depletion column was separated by 2-DE (D). Albumin is the predominant protein present (around 67 kDa), but there is the suggestion of additional proteins having been removed from the whole lymph**.

**Figure 3.2**

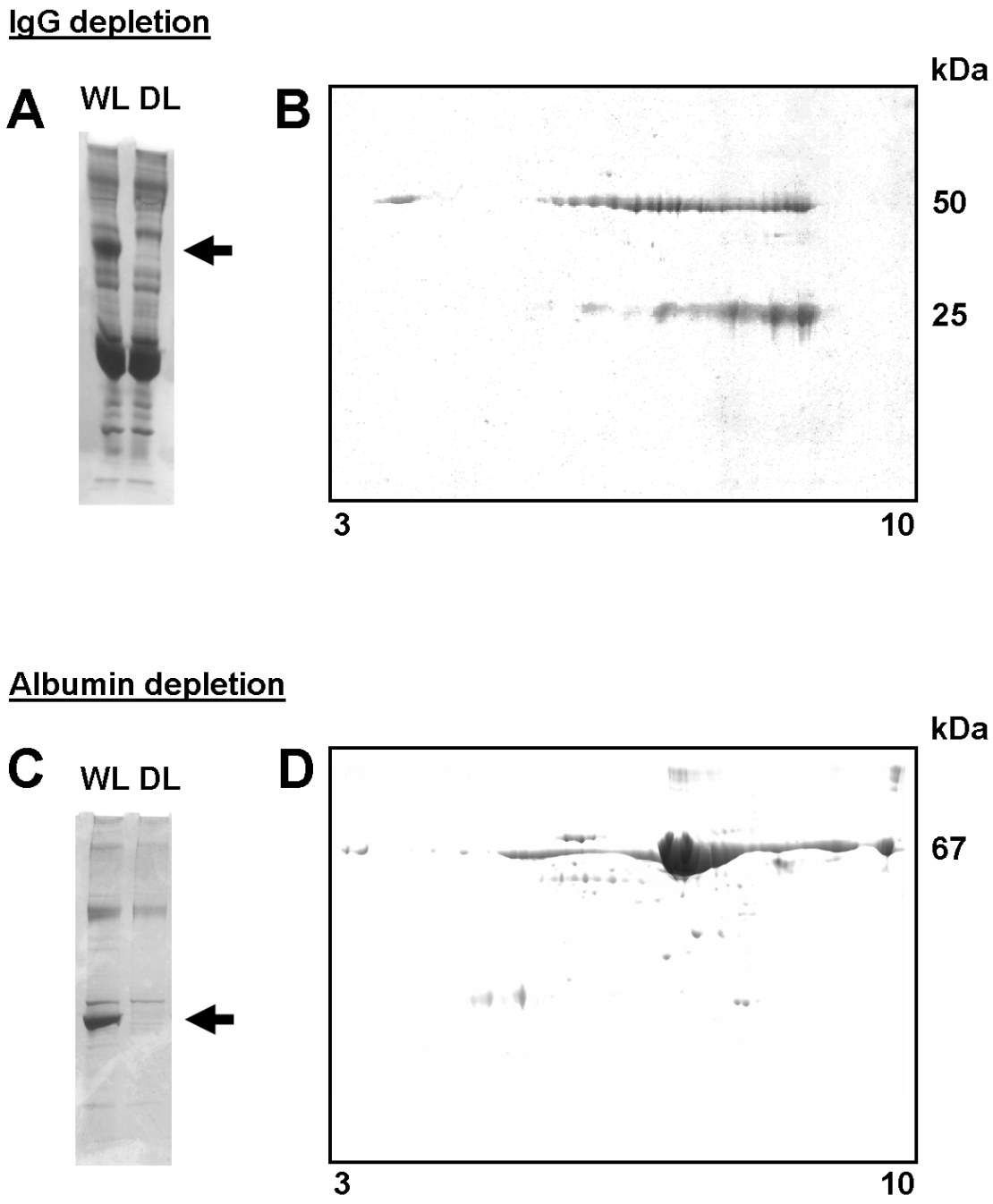

IgG depletion
A
WL DL
B
kDa
50
25
3
10
Albumin depletion
C
WL DL
D
kDa
67
3
10

**Table 3.1 : Proteins identified by LC-ESI-MS/MS which have been removed from lymph by albumin and immunoglobulin depletion columns.**

| Gene name | Protein name | Accession no. | MASCOT score | Theoretical PI | Theoretical MW | No. peptides matched | Sequence coverage (%) |
|---|---|---|---|---|---|---|---|
| *Immunoglobulin co-depletion* | | | | | | | |
| CO4_BOVIN | Complement C4 precursor (*Bos taurus*) | P01030 | 47 | 6.15 | 103018 | 2 | 5 |
| RBP2_HUMAN | Ran-binding protein 2 *(Homo sapiens*) | P49792 | 43 | 5.85 | 362365 | 11 | 1 |
| | | | | | | | |
| *Albumin co-depletion* | | | | | | | |
| H4_BOVIN | Histone H4 (*Bos taurus*) | P62805 | 96 | 11.36 | 11229 | 4 | 29 |
| TTHY_SHEEP | Transthyretin precursor (*Ovis aries)* | P12303 | 74 | 5.63 | 15875 | 4 | 24 |

**The above table excludes albumin, and the contaminants keratin and trypsin.**

**For MASCOT scores >38, $p<0.05$**

## *3.3 Optimisation*

### 3.3.1 IPG strip pH range

A preliminary trial was carried out to assess the optimal pH range to be used in the IPG strips. Further details of the principles involved in 2-DE can be found in Materials and Methods (section 2.4.2). The aim was to choose the range giving maximum coverage of the proteome, whilst still producing sufficient spot separation to allow further downstream analysis. pH 4-7, 3-10 and 3-10NL were all assessed. pH 4-7 and pH 3-10 NL were found to give the best spot separation in the more neutral pH ranges, which is where the majority of proteins in blood and lymph lie. pH 3-10 IPG strips, although giving good coverage of the whole proteome, including at the more extreme pH ranges, were found to give inadequate individual spot separation between pH 5 and 8, as there was apparent overloading of proteins in these areas. This study showed the optimum pH range of IPG strip to be used was pH 3-10NL. Figure 3.3 illustrates the difference in spot pattern between the different pH ranges.

### 3.3.2 Staining technique

Having optimised the running conditions, a comparison of staining methods was then carried out. Figure 3.4 shows a silver stained gel (A) and an equivalent Colloidal Coomasie Blue (Proteomic Solutions) stained gel (B).

As expected, the silver staining technique was more sensitive, detecting a greater proportion of protein on the gel. However, due to the multitude of spots and the background staining which occurred with silver staining, the Colloidal Coomasie Blue stained gel actually produced a clearer gel and still showed an adequate number of spots to be of use. Due to the nature of this study, with spot identification and spot change being of priority, the Colloidal Coomasie Stain was deemed to be optimal as it would allow individual spots to be studied and excised for identification, with the acceptance that some sensitivity would be sacrificed. .

**Figure 3.3 IPG strip pH range optimisation**

**Gel image (A) shows separation of lymph using an IPG range of pH 4-7. Gel image (B) shows separation of lymph using an IPG range of pH 3-10NL. MW shown in KDa.**

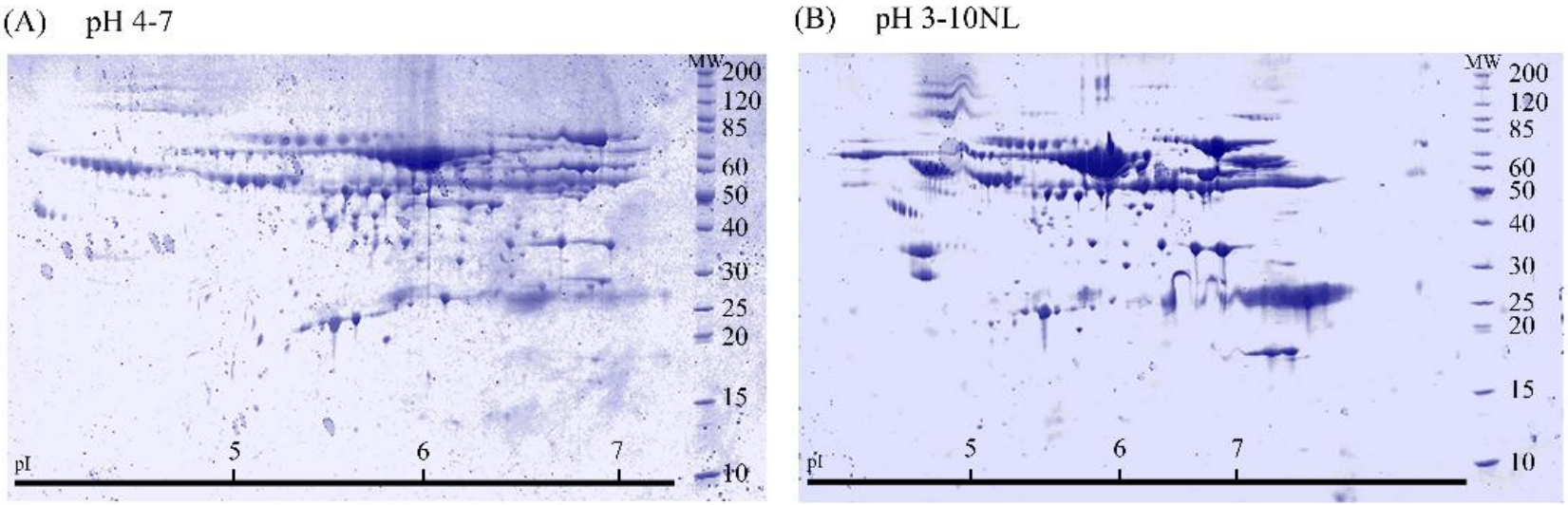
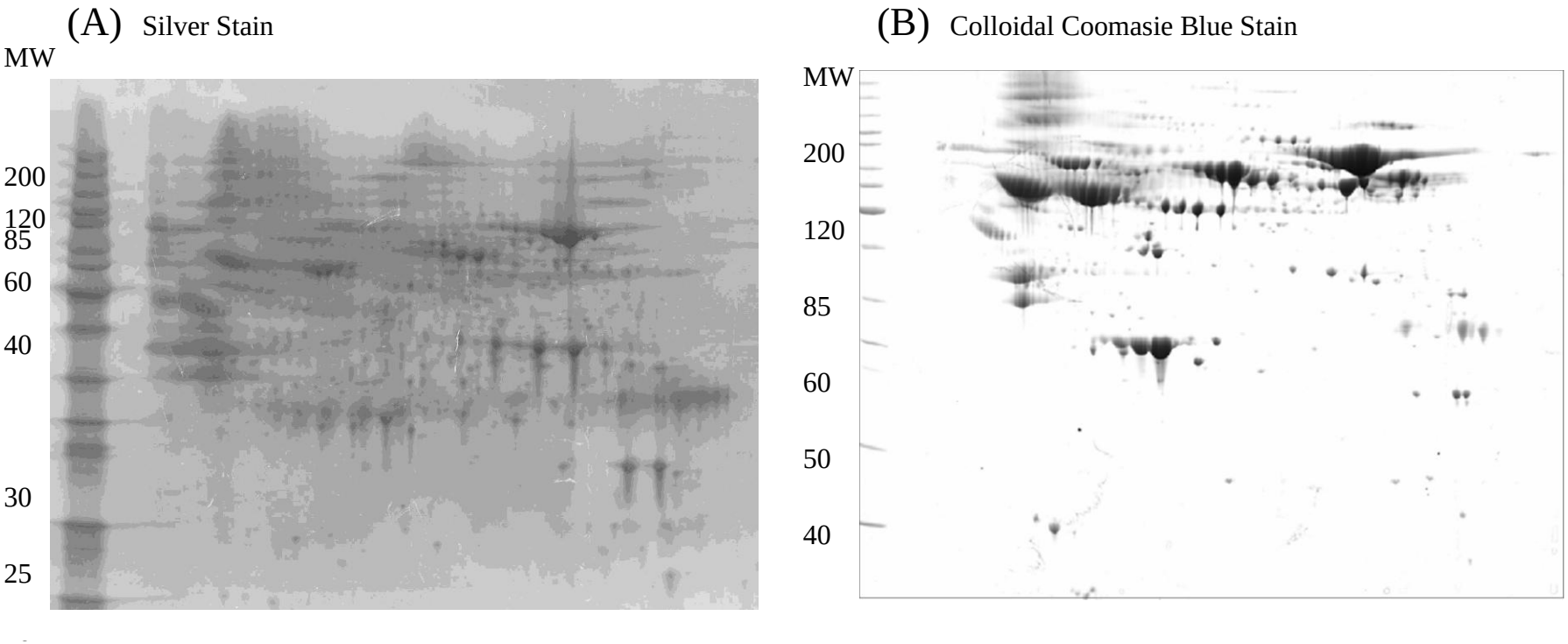


**Figure 3.4 Comparison of lymph stained using either silver stain or Colloidal Coomasie Blue Stain. Gel image (A) shows lymph (depleted of IgG and albumin), separated using pH 3-10NL IPG strip, and stained using silver stain. Gel image (B) shows lymph (depleted of IgG and Albumin), separated using pH 3-10NL IPG strip, and stained using Colloidal Coomasie Blue Stain (Proteomic Solutions). MW shown in KDa.**



## *3.4 Repeatability*

Figure 3.5 shows the scanned images from five gels, from different animals, all on Day 0 of a primary infection. It was found that the technique created a similar pattern of spots each time, such that the spots could be matched and comparisons made between gels. The repeatability of the technique is also verified by the relatively small error bars that results from analysis of the gel data (Figure 3.7).

## *3.5 2-DE and MALDI-TOF*

Further information regarding MALDI-TOF analysis can be found in Materials and Methods (section 2.7). Separation of the depleted lymph samples, on average, yielded 132 different protein spots or clusters of spots. As found previously in blood and lymph plasma (Leak *et al.* 2004), many proteins exist in multiple charged forms of similar MW. A total of 87 separate spots were cut from Coomassie stained gels and subjected to MALDI-TOF analysis and subsequent peptide mass fingerprint searches within the available databases. This provided information as to the identity of 38 individual spots and 25 unique proteins (Figure 3.6 and Table 3.2). A significant MASCOT score, where $p<0.05$, was used to determine reliable protein identification. These were then validated by cross-checking the pI and MW of the predicted protein, against that of the spot actually excised from the gel. The absence of a comprehensive ovine database hampered protein identification; an issue highlighted previously by Leak et al (Leak *et al.* 2004). However, this study was able to identify by MS, a number of proteins in lymph which had previously only been identified by gel matching with gels of vertebrate blood plasma, including plasminogen, α-1 antitrypsin, hemopexin, α-1 β glycoprotein, α-2 HS glycoprotein and haptoglobin. In addition, it identified a number of proteins, not previously identified in lymph, including retinol binding protein, gelsolin, apolipoprotein A-IV, tetranectin and the acute phase protein, serum amyloid A. However, this study failed to identify glial fibrillary astrocyte acidic protein, one of the proteins identified by Leak et al. to be exclusive to lymph plasma when compared to

blood plasma. This current study also failed to identify neutrophil cytosol factor 1, which was shown to constitute multiple spots in the previous analysis of the lymph proteome.

**Figure 3.5: Technique repeatability.**

**Shown below are Day 0 gels from 5 different, primary infection animals. All show a similar spot pattern.**

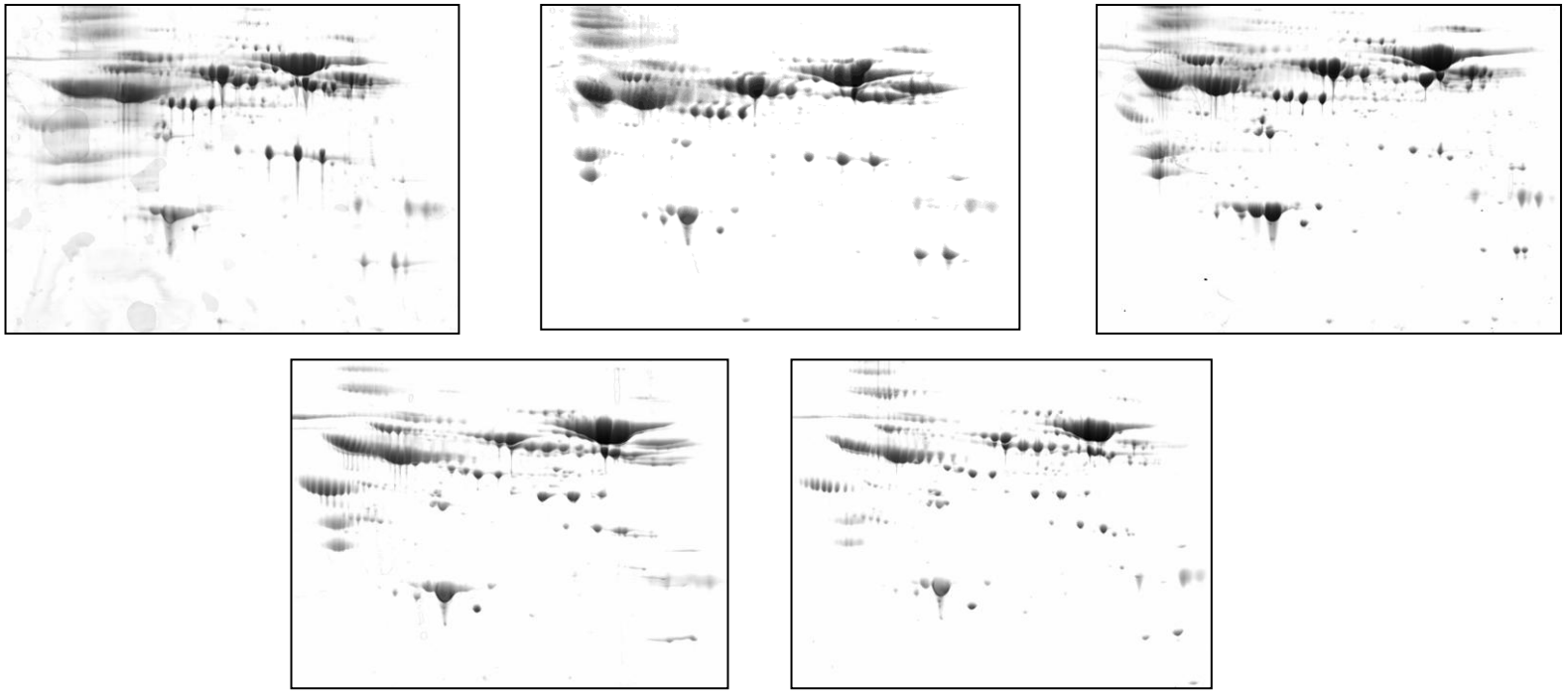


**Figure 3.6: 2-DE of albumin and IgG-depleted lymph. The equivalent of 80 µl gastric lymph was depleted of albumin and IgG (see Materials & Methods) and separated by 2-DE. The image is annotated with the gene names of the proteins identified by MALDI-TOF and MS/MS. Refer to Table 3.2 for details. (This image is representative of 30 individual gels.)**

3

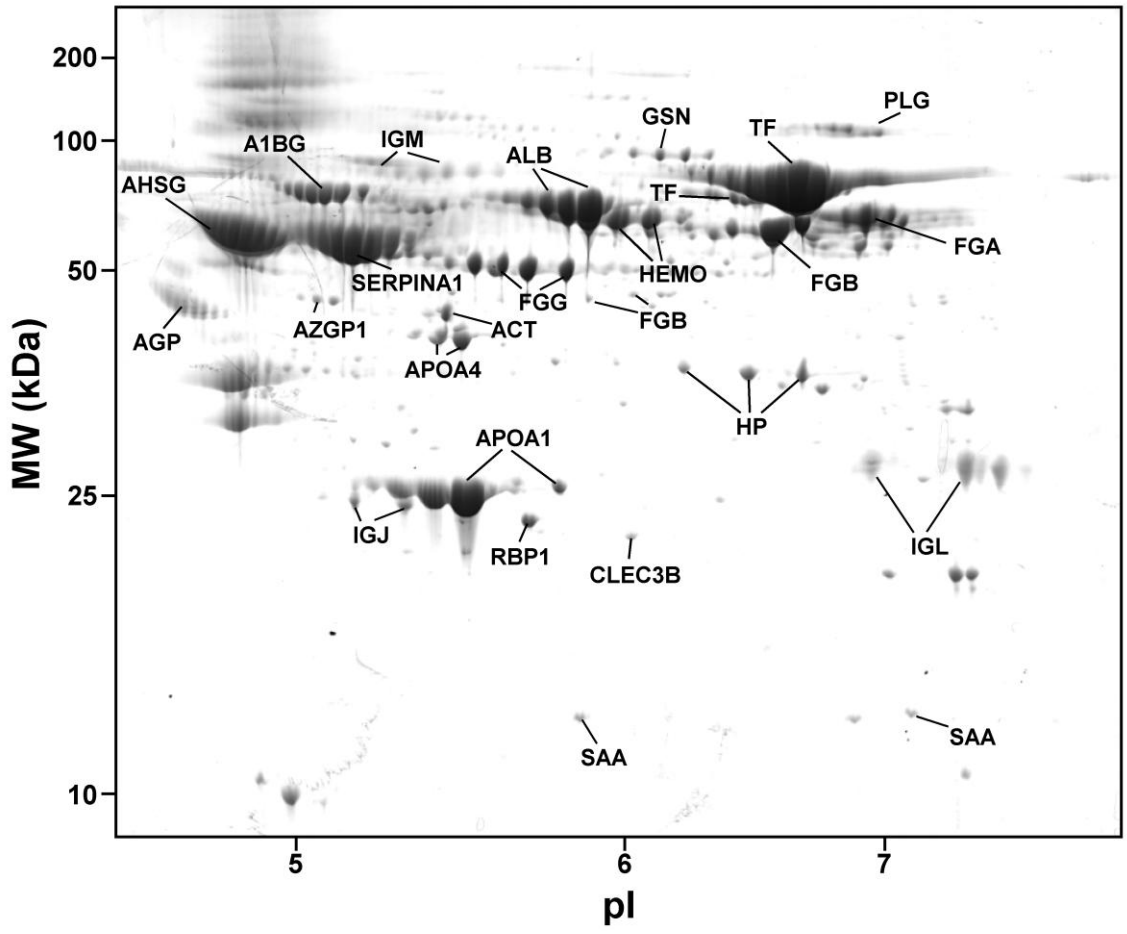


**Table 3.2 : Mass Spectrometry Results**

| Gene name | Protein name | Accession no. | MASCOT score | Theoretical PI | Theoretical MW | Values/queries matched (unmatched) | Sequence coverage (%) |
|---|---|---|---|---|---|---|---|
| A1BG | α-1 B glycoprotein (*Bos taurus*) | AAI05375 | 113* | 5.29 | 53520 | 10 (54) | 20 |
| ACT | Actin-2 (*Bos taurus*) | P61157 | 68* | 5.31 | 41637 | 10 (58) | 29 |
| AGP | α-1 acid glycoprotein (*Bos taurus*) | AAI02741 | 187* | 5.62 | 23168 | 8 (60) | 37 |
| AHSG | α-2 HS glycoprotein (*Ovis aries*) | P29701 | 113 | 5.17 | 38655 | 12 (50) | 44 |
| ALB | Serum albumin (*Ovis aries*) | P14639 | 82 | 5.80 | 69143 | 25 (51) | 42 |
| APOA1 | Apolipoprotein A-1 (*Bos taurus*) | P15497 | 116 | 5.57 | 28415 | 15 (62) | 50 |
| APOA1 | Apolipoprotein A-1 (*Bos taurus*) | P15497 | 125 | 5.57 | 28415 | 14 (76) | 52 |
| APOA4 | Apolipoprotein A-IV (*Bos taurus*) | Q32PJ2 | 130 | 5.30 | 42991 | 16 (29) | 43 |
| APOA4 | Apolipoprotein A-IV (*Bos taurus*) | Q32PJ2 | 143 | 5.30 | 42991 | 18 (35) | 45 |
| AZGP | Zinc-α 2-gylcoprotein (*Bos Taurus*) | D21058 | 189* | 5.13 | 33830 | 11 (45) | 37 |
| CLEC3B | C-type lectin (sim. to tetranectin) (*Bos taurus*) | Q2K157 | 87 | 5.47 | 22130 | 7 (23) | 42 |
| FGB | Fibrinogen β chain precursor (*Bos taurus*) | P02676 | 103 | 8.19 | 27621 | 16 (69) | 56 |
| FGG | Fibrinogen gamma-B chain precursor (*Bos taurus*) | AAI02630 | 74* | 5.54 | 50212 | 9 (78) | 23 |
| FGG | Fibrinogen gamma-B chain precursor (*Bos taurus*) | AAI02630 | 85* | 5.54 | 50212 | 8 (57) | 14 |
| GSN | Gelsolin (actin depolymerising factor)horse | Q28372 | 193* | 5.58 | 80646 | 18 (42) | 28 |
| HP | Haptoglobin (*Bos taurus*) | AAI09669 | 64 | 7.83 | 44831 | 9 (36) | 19 |
| HP | Haptoglobin (*Bos taurus*) | AAI09669 | 77 | 7.83 | 44831 | 14 (82) | 29 |
| HP | Haptoglobin (*Bos taurus*) | AAI09669 | 80 | 7.83 | 44831 | 13 (63) | 43 |
| IGJ | Immunoglobulin J chain(*Bos taurus*) | AAI03427 | 53 | 5.10 | 18359 | 5 (36) | 32 |
| IGL | Immunoglobulin lambda chain (*Bos taurus*) | AAK84156 | 54 | 8.46 | 11305 | 4 (28) | 66 |
| IGL | Immunoglobulin lambda chain (*Bos Taurus*) | AAK84156 | 46 | 8.46 | 11305 | 5 (26) | 77 |
| IGL | Immunoglobulin lambda chain (*Bos taurus*) | AAK84156 | 56 | 8.46 | 11305 | 5 (45) | 77 |
| IGM | Immunoglobulin mu chain (*Ovis aries*) | AAA51379 | 145 | 5.47 | 64480 | 15 (37) | 39 |
| HEMO | Similar to hemopexin (*Bos taurus*) | AAI02688 | 191* | 7.90 | 52176 | 13 (63) | 17 |
| PLG | Plasminogen (*Ovis aries*) | P81286 | 83 | 7.54 | 38664 | 10 (65) | 36 |
| RBP1 | Retinol binding protein (*Bos taurus*) | AAX46484 | 70 | 5.26 | 20158 | 13 (51) | 70 |
| SAA | Serum amyloid A (*Bos taurus*) | P35541 | 97* | 6.14 | 12552 | 10 (73) | 81 |
| SAA | Serum amyloid A (*Bos taurus*) | P35541 | 80* | 6.95 | 12595 | 5 (40) | 29 |
| SERPINA1 | α-1 antitrypsin (*Ovis aries*) | ABF57415 | 100 | 5.83 | 45956 | 18 (82) | 40 |
| TF | Transferrin (*Bos taurus*) | AAI22603 | 68 | 6.75 | 77703 | 16 (56) | 43 |
| TF | Transferrin (*Bos taurus*) | AAI22603 | 49 | 6.75 | 77703 | 21 (70) | 32 |

a) * indicates a combined MALDI-TOF and MS/MS score b) for MASCOT scores > 69, $p<0.05$ c) all values are for PMF searched with the fixed modification carbamidomethyl and peptide tolerance of 50 ppm.

## *3.6 2-DE gel analysis*

Densitometric analysis of these gels revealed many proteins which changed greater than 1.5-fold during the course of infection in an individual animal. However, when the results are combined (n=5) for each time point, only one protein in the animals undergoing a primary infection, and two proteins in immune animals undergoing challenge infection, were found to change significantly by Linear Mixed Model analysis (repeated measures) over the time course of experimental infection. This may be due to individual variation consistent with the sheep belonging to an outbred population. Summary statistics of this analysis and hypothesis testing for the significantly changed proteins is shown in Appendix 1.

In the sheep undergoing a primary infection, gelsolin, an actin-depolymerising protein, was found to decrease significantly ($p<0.05$) (Figure 3.7 (A)). This reduction was significant between days 0 and 21. These results were further verified by means of Western Blotting (Figure 3.7 (A) and see Chapter 5). All five animals showed this decrease between day 0 and 11, but only two continued to decrease between days 11 and 21 of infection. This could indicate a transient effect of *T.circumcincta* infection on gelsolin in lymph plasma. No similar trends were evident in the immune animals between day 0 and day 10.

In immune animals undergoing a challenge infection with *T.circumcincta*, α-1 β glycoprotein and hemopexin were changed significantly in challenged, compared to non-challenged time-points.

Hemopexin, involved in haem scavenging and transport, was significantly upregulated in immune animals at day 10 following a challenge dose of *T.circumcincta* (Figure 3.7 (B)). Four out of five animals showed an increase by day 6, and four out of five animals increased between days 6 and 10.

α-1 β glycoprotein, a protein of unknown function, was significantly down-regulated in immune animals in the 10-day period following challenge (Figure 3.7(C)). This decrease was significant at day 10 compared to day 0, and at day 10 compared to day

6. Four out of five animals showed a reduction by day 6, but this was not statistically significant. All five animals showed a reduction by day 10. As mentioned previously, immune exclusion of the worms will have already occurred by this time hence it is unlikely that α-1 β glycoprotein has a role in this process. The significant changes in lymph, as detected by 2-DE gel analysis are summarised in Table 3.3.

Another group of proteins, the acute phase proteins (specifically serum amyloid A (SAA), haptoglobin), visually appeared to be decreasing with infection in some animals, especially in the primary infection group. However, the timing of this decrease was inconsistent between animals, with some having the lowest level at day 11, and others having the lowest level at day 21 (means and SEM can be found in appendix 1, table 1 and 2 , individual animal data is shown in interaction plots, appendix 1, section A6, and see Chapter 4). As a result, no statistically significant change was recorded. It did indicate however that the abundance of these proteins did vary throughout infection.

**Figure 3.7: Expression of selected proteins in lymph from sheep infected with *T.circumcincta.* Panels A-C show examples of gel images and bar charts representing the combined data from 2-DE gel densitometry analysis for gelsolin, hemopexin and alpha-1-B glycoprotein, respectively (+/-SEM). Gelsolin decreased significantly in expression level during primary infection (PI) (A). At day 21, the level of gelsolin detected was significantly lower than at day 0 (*; $p<0.05$). The panels above show the row of spots identified as gelsolin (arrow) on day 0, and the same area on day 21, from the same animal. The inset image is a western blot using anti-gelsolin antibody, showing a decline in gelsolin expression in lymph at day 21. This blot is representative of blots from 5 animals. Hemopexin increased significantly (*; $p<0.05$) between day 0 and day 10 in immune challenged (IC) animals (B). Example hemopexin spots are indicated (arrow) at day 0 and day 10 in gels from the same animal. Albumin remained in the samples to varying degrees and was consistently identifiable on all gels (arrowhead). Alpha-1 B glycoprotein decreased significantly (*; $p<0.05$) in immune challenged (IC) animals between day 0 and day 10(C). Alpha-1 B glycoprotein spots are indicated (arrow) in day 0 and day 10 gels from the same animal. (The magnified gel images are each representative of 5 individual gels.)**

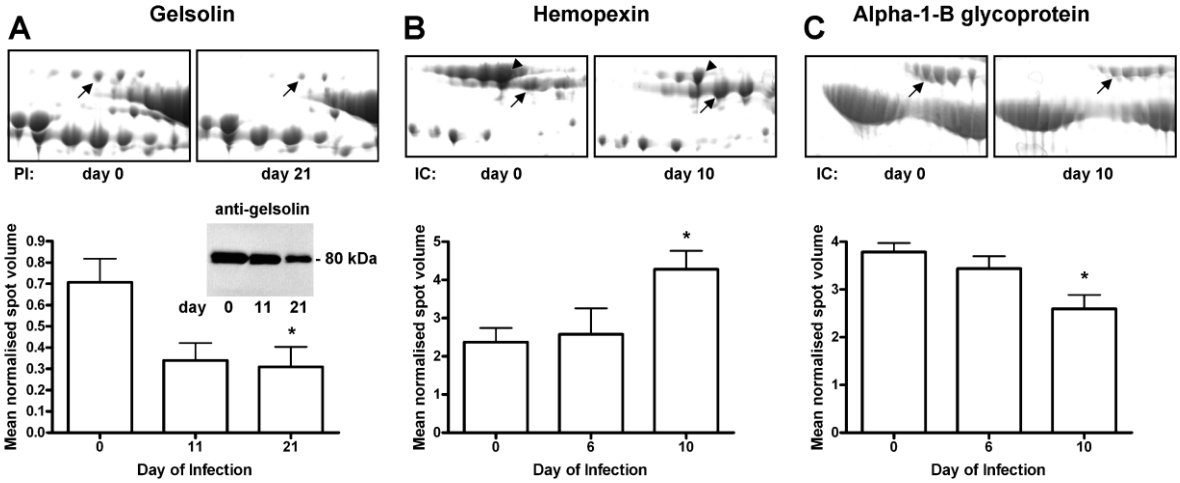



**Table 3.3 : Summary of significant changes**

| Protein | Infection Group | Observed significant change |
|---|---|---|
| Gelsolin | Primary Infection | Decrease (days0-21) |
| Hemopexin | Immune Challenged | Increase (days 0-10) |
| α-1 B glycoprotein | Immune Challenged | Decrease (days 0-10) |

## *3.7 Discussion*

In this study lymph, formed from the interstitial fluid bathing a diseased organ, was collected with the aim of identifying potentially important local effector molecules, involved in either specific pathology or in the host immune response to infection with *T. circumcincta.* Previous studies (Smith *et al.* 1987b) have shown substantial changes in the cellular composition of lymph as a result of infection, and one of the questions which this current study wished to address was whether the lymph proteome was equally altered in disease.

Relatively little is known regarding the proteome of efferent lymph, despite the many other studies of lymph both from normal and from infected tissues, which have mainly dealt with the cellular composition of lymph. Leak et al. (2004) described the proteome of sheep mediastinal lymph, which drains the chest cavity, but the dynamics of the lymph proteome in disease has not previously been investigated.

In carrying out this study, a strategy of depleting the two most abundant proteins was employed. Immuno-depletion of the most abundant proteins has been applied widely in the study of the human plasma proteome (Omenn *et al.* 2005). However, limited reagents are available for sheep. The pre-fractionation methods employed in this series of experiments successfully depleted the two most abundant lymph proteins, allowing greater protein loading at the IEF stage. Relatively more of the low abundance proteins could be loaded, improving the chance of successful isolation and identification of these proteins.

The specificity of depletion was investigated jointly by means of 2-DE and also LC-ESI-MS/MS. Both columns were found to be suitably specific. The IgG column inadvertently removed only two additional proteins – complement C4 and human ran binding protein 2. This differed from the findings of Echan et al, who identified multiple non-specific bands on 1D gels of elution fractions from the Protein G depletion kit (Echan *et al.* 2005). However, the removal of complement C4 may

potentially have masked interesting innate immune response data which may otherwise have been detected on 2-D gel analysis. The column used for albumin removal was also found to be quite specific. This level of specificity is consistent with the findings of Hinerfeld et al. when using this method to deplete human and rat blood plasma (Hinerfeld *et al.* 2004).

A number of proteins were identified by mass spectrometry which had not previously been identified in lymph by this methodology (Table 3.2). These included multiple charged forms of α-1 acid glycoprotein and α-1 B glycoprotein, two distinct forms of serum amyloid A and apolipoprotein A-1, and actin. It is known from previous studies, that proteins of local origin, including mucosally-derived pepsinogen and IgA are present and increase substantially during infection, with the result that they are detected in higher concentrations in lymph compared to blood (Smith *et al.* 1987b). The failure to detect pepsinogen probably reflects the limited sensitivity achievable by this method, despite the elimination of the two most abundant proteins, and this sensitivity will be discussed later. Nevertheless, as mentioned above, a number of proteins that had not been described in efferent lymph previously were identified.

In contrast to mediastinal lymph analysis (Leak *et al.* 2004), this study failed to identify glial fibrillary astrocyte acidic protein and neutrophil cytosol factor on 2-D gels. There may be several reasons for this. In the previous study, mediastinal lymph was apparently collected for analysis immediately following surgery, which is known to be associated with a transient neutrophilia (Heath *et al.* 1962). In this study, all samples of normal lymph were always collected at least 24 hours after cannulation, so that the early flush of neutrophils (often more than 50% in the first 24 hours (Hall & Morris 1962; Spencer & Hall 1984)) had dispersed. The cellular content of the lymph at this stage has been shown to be more than 99% small/medium lymphocytes (Haig *et al.* 1999). The absence of neutrophil cytosol factor in the present study is therefore likely due to the relative absence of neutrophils in efferent lymph 24 hours after surgery, neutrophils being the predominant source of this protein (Lomax *et al.* 1989). Leak et al. (2004)

hypothesised that the glial fibrillary astrocyte acidic protein was of local origin, as shown by its absence in blood plasma and presence in lymph plasma. Since lymph is essentially a composite of the interstitial tissues which it drains, the different tissue source in the previous study may account for the variations observed in the proteome (mediastinal cf. gastric lymph). However, as the sequence identified pertaining to this protein (LALDIEIATYR) is also present in human keratin, this could alternatively be an inadvertent artifact of contamination. This peptide was identified in LC-ESI-MS/MS analysis of the co-depletion samples in the current study. No other peptide corresponding to glial fibrillary astrocyte acidic protein was identified, and therefore it falls below the criteria for identification.

Using lymph proteomics to monitor the kinetics of the response to *T.circumcincta*, significant changes in the level of several proteins in the lymph draining the gastric mucosa were detected. Firstly, gelsolin decreased substantially between days 0 and 11, and days 11 and 21 ($p<0.05$, between days 0 and 21). This trend highlighted by 2-DE gel analysis was then further verified by means of western blotting (Figure 3.7(A)). Gelsolin is an actin scavenging protein (Sun *et al.* 1999a) present in both secreted and cytoplasmic forms (Yin *et al.* 1984). Secreted plasma gelsolin has the role of binding free actin, such as may be released from damaged cells, in order to prevent actin polymerisation and hence detrimental alterations to plasma viscosity. It is possible that actin-gelsolin complexes form when actin is released from the worm-damaged mucosa. If the rate of removal of these complexes exceeds the rate of gelsolin production, then a reduction in concentration will result.

Gelsolin is also known to be down-regulated as cells become less differentiated (Vandekerckhove *et al.* 1990). Replacement of acid producing parietal cells, with undifferentiated, rapidly dividing, non-acid secreting cells, is a well recognised histological feature of the pathology caused by this parasite during invasion of the abomasal gland (Simpson 2000; Urquhart *et al.* 1996). It may be that the kinetics of gelsolin, demonstrated in lymph, are indeed a reflection of the local response to infection with *T.circumcincta*.

Furthermore, gelsolin is known to alter the viscosity of airway mucus (Vasconcellos *et al.* 1994b), and may also have a role in the regulation of mucin secretion from goblet cells (Ehre *et al.* 2005). Intestinal mucus in thought to play an important role in the expulsion of gastro-intestinal parasitic nematodes (Miller 1987). It is therefore possible that the local gelsolin levels not only represent pathology caused by *T.circumcincta*, but may also be part of the host response to infection.

Surgical cannulation, unavoidable in the collection of gastric lymph, may have itself influenced gelsolin levels. Trauma in other species has been shown to reduce this protein in blood (Christofidou-Solomidou *et al.* 2002). In the current study, the absence of a similar trend in the immune group makes this explanation less plausible. However, lymph was collected for a shorter period in the immune group (10 days) and so a surgical cause can not be ruled out. Gelsolin was chosen as a candidate for further analysis (see Chapter 5).

Hemopexin increased in the immune challenged animals only. This protein binds haem and transports it to the liver for iron recovery. Hemopexin is known to be an acute phase protein, with its liver synthesis being up-regulated in response to pro-inflammatory cytokines. The increase in hemopexin may be due to surgically induced inflammation, though again the observed change is not consistent between different infection groups, and so this is less likely. Hemopexin also has a role in innate immunity by reducing iron availability to bacteria (Ascenzi *et al.* 2005; Rocha *et al.* 2001). It may be upregulated to provide innate antibacterial activity in the face of the Th-2 driven anti-nematode response.

The final protein found by gel analysis to change significantly, was α-1 B glycoprotein. There is little previously published regarding this protein, and its function remains unknown. This protein was found to be reduced during challenge of immune animals, but its role in either immunity to the parasite, or in the pathology of the disease, remains unclear.

Proteomic analysis of lymph failed to identify other proteins, known to be present in lymph and thought to be of local origin. Pepsinogen has been previously identified

in lymph from animals infected with *T.circumcincta* (Smith *et al.* 1983b) and was identified in the samples analysed in this study by means of an activity assay (data not shown). Although not identified in the LC-ESI-MS/MS study, it is possible that pepsinogen was removed during depletion as whole lymph samples were used in the activity assay. It would have been appropriate to repeat the assay using depleted lymph, but given the time-consuming nature and the small volumes involved in depletion methods, this was not done. Intelectin has also been shown to be locally transcribed in the abomasum, with expression being unregulated following infection with *T.circumcincta* (French *et al.* 2008). Intelectin was identified in the depleted lymph samples analysed in this study by means of western blot (data not shown).

In conclusion, the proteomic study of lymph has the potential to give new insights into local responses to infection. Using 2-DE separation, a semi-quantitative analysis of changes to the lymph proteome during the course of a disease state was demonstrated for the first time. Analysis by the 2-DE methods used, even with removal of the two most abundant proteins, proved to be too insensitive to detect locally produced proteins, which have been shown by other methods to be present. More sensitive means of separating out the components of this complex proteome are required in order to take full advantage of the information this tissue fluid has to offer.

As a result of this study, the acute phase proteins haptoglobin, serum amyloid A (SAA) and alpha-1 acid glycoprotein and also gelsolin were selected for further detailed analysis.

# 4 Chapter 4: The Acute Phase Protein response to *Teladorsagia circumcincta*

## *4.1 Introduction*

Studying the lymph proteome by means of 2-DE identified three of the main ruminant acute phase proteins (APP) by MALDI-TOF in lymph (Chapter 3). These proteins are haptoglobin, serum amyloid A (SAA) and alpha-1 acid glycoprotein (AGP). The kinetics of these proteins did appear to vary throughout the experimental period although the changes did not reach significance. Therefore, in this study, the acute phase response (APR) to *T. circumcincta* was examined more closely.

The APR is associated with a variety of physiological and biological changes which upset the normal homeostatic state of the healthy animal including pyrexia (Baracos *et al.* 1983; Dinarello 1983; Dinarello & Bunn, Jr. 1997), reduced appetite, (Gruys *et al.* 2005) and changes in capillary permeability. In addition, the levels of some important inflammatory mediators released from activated inflammatory cells promote the acute phase reaction (Ceciliani *et al.* 2002). The changes which occur as a result of this APR act to assist the body in repairing and/or isolating the insult and hence speed the process of healing and a return to the normal healthy state. In response to insult, local inflammatory cells secrete a number of cytokines into the bloodstream which signal the liver to produce a number of acute phase proteins. Also, production of some proteins is down-regulated.

The local response at the site of insult is followed by systemic signalling of the response by means of the spread of locally produced cytokines such as interleukin 1 (IL-1), interleukin 6 (IL-6) and tissue necrosis factor alpha (TNF-α). These alter the level of transcription and translation of certain genes within hepatocytes (with the liver being the principal site of acute phase protein synthesis in the mammalian body (Heinrich *et al.* 1990; Heinrich *et al.* 1998; van Miert 1995)). The plasma proteins whose synthesis is either up- or down-regulated in this way are known as Acute Phase Proteins (APPs). APPs can be either negative, whereby synthesis is reduced in

response to the pro-inflammatory cytokines or positive, where synthesis is increased in light of the systemic acute phase response (Dinarello 1983; Ingenbleek & Young 1994).

Bacterial infections are usually accompanied by a significant APR, thought to be due to release of IL-1β, and TNF-α by cells of the mononuclear-phagocyte lineage in response to endotoxin (Werling *et al.* 1996). This has been shown to be true of ruminants, with calves showing a distinct acute phase protein response (APPR) following challenge with either *Pasteurella haemolytica* or endotoxin (Conner *et al*. 1989). The APR in viral infections is often less severe, with IFNγ being the principle cytokine released from the mononuclear inflammatory cells involved in the immune response (Hofner *et al*. 1994)**.**

In parasitic infection the literature is contradictory, with some infections appearing to induce a significant acute phase response, and others not, even when the species of parasite involved are closely related. The tropical tick-borne protozoal parasite, *Theileria annulata,* produces a significant systemic APPR, more so in less resistant breeds such as *Bos taurus*, compared to the more resistant indigenous breed, *Bos indicus* (Glass *et al.* 2005; Glass *et al.* 2003), and is believed to be directly correlated to disease pathology. Similarly, another vector borne protozoan parasite, *Trypanosoma cruzi* also elicits a distinct SAA3 response in the host (Ferreira *et al.* 1999) during the acute phase of Chagas Disease. Protozoan parasites differ from nematode parasites in having a far more significant parasitaemic phase.

Acute phase protein responses to the nematode parasites are more variable. Cooper et al. (1997) reported a positive systemic APPR in children naturally infected with *Trichuris trichuria* (Cooper *et al*. 1997). This is a nematode parasite with a life-cycle similar to that of *T.circumcincta,* being confined solely to the gastrointestinal tract without any tissue migratory phase. In contrast, it has been suggested that nematode induced intestinal pathology alone is not a stimulus for a systemic APPR, as shown by the absence of such when intestinal *Nippostrongylus brasilienis* infection in the rat is compared to the tissue migratory phase *of N. brasiliensis* (Stadnyk *et al.* 1990).

This study also compared the APPR to *Trichinella spiralis* with that of *N. brasiliensis* and found that, despite similar intestinal pathologies, *T.spiralis* did not elicit an APPR, adding further weight to the hypothesis that intestinal inflammation alone during parasite infection is insufficient to stimulate the APPR.

There has been little attention to APPR in ruminant nematode infections but Conner et al. (1989) showed that a proportion of calves challenged with the abomasal nematode *Ostertagia ostertagi* (a close relative of *T. circumcincta*) showed an increase in serum acute phase protein levels (haptoglobin, α-1 proteinase inhibitor, seromucoid and ceruoplasmin) but, in those that did, the response was very variable both in terms of magnitude and timing (Conner *et al.* 1989). The three APP shown to be present in lymph (Chapter 3), namely SAA, AGP and haptoglobin, all have the potential to modulate the immune response to a parasite. SAA is thought to be important in HDL transport of cholesterol (Coetzee *et al*. 1986). During the acute phase response, this function may be important in the removal of cholesterol from mononuclear cells and dead cells at the site of inflammation (Lindhorst *et al*. 1997). SAA has also been shown to have chemoattractant activity for cells of the immune system, including monocytes, neutrophils and T-cells (Badolato *et al.* 2000; Xu *et al.* 1995). SAA can also regulate expression of cell-surface markers involved in adhesion and bacterial recognition, as well as inducing release of lactoferrin from PMN (Badolato *et al.* 2000), which aids phagocytosis, suggesting SAA may help accentuate the immune functions of these cell types. SAA can inhibit the oxidative burst of neutrophils (Linke *et al*. 1991). Finally, bovine and human mammary associated SAA (M-SAA3) can promote the transcription of the intestinal mucin *MUC3* gene in human intestinal cells in vitro (Larson *et al.* 2003), mucin responses also being central to preventing establishment and clearing gastrointestinal nematode infection (Miller 1987).

AGP can inhibit platelet aggregation (Costello *et al.* 1979), and reduce tissue damage, both locally and systemically following ischaemic damage of the intestine in the rat (Williams *et al*. 1997). It also inhibits the phagocytic activity of neutrophils (Laine *et al.* 1990; van Oss *et al.* 1974) and the oxidative burst (Laine *et al*. 1990).

AGP has been shown to actually prolong the life of blood derived bovine monocytes *in vitro* by a reduction in the rate of apoptosis, and hence may enhance the immune response (Ceciliani *et al*. 2007).

The main function of haptoglobin is to bind free haemoglobin, released from erythrocytes. By forming this complex with haemoglobin, haptoglobin allows access of degradative enzymes to the haemoglobin, and also prevents renal loss of iron and kidney damage by haemoglobin (Dobryszycka 1997). Haptoglobin can also bind to various immune cells, including granuolocytes, monocytes, CD8+ T cells and NK cells and hence can modulate the immune response to a pathogen. The effects on the individual cells is wide ranging inhibiting lymphocyte and B cell proliferation in the high concentrations typically seen during the APR (Baseler & Burrell 1983; Samak *et al.* 1982). It inhibits superoxide production during the respiratory burst in neutrophils (Oh *et al.* 1990), and causes an increase in intracellular calcium in this cell type. Prostaglandin E, which has the potential to influence vascular smooth muscle cells and platelet aggregation, is released in greater amounts from alveolar macrophages when stimulated with haptoglobin (Baseler & Burrell 1983). The haptoglobin gene has been shown to be expressed in alveolar macrophages and eosinophils in diseased lung tissue, but transcription was not evident in normal lung tissue (Yang *et al*. 2000). In addition, haptoglobin levels are known to be lower in human patients suffering from allergic type diseases such as rhinitis and asthma (Piessens *et al.* 1984). El-Ghmati et al. (2002) found that haptoglobin bound strongly *in vitro* to the human mast cell line HMC-1 (El-Ghmati *et al.* 2002). Haptoglobin can also inhibit cysteine proteases, extensively produced and secreted by parasitic helminths (Pagano *et al.* 1982; Snellman & Sylven 1967). This is of possible relevance here with the most prominent protein secreted by *T. circumcincta* L3 in the earliest days of infection ( 1, 3 and 5 days post infection) being a Cathepsin F cysteine protease which is immunogenic eliciting a pronounced IgA response (Smith *et al*. 2009).

As mentioned previously, the APPR is initiated by the pro-inflammatory cytokines IL-1, IL-6 and TNF-α. Although it is well established that the response to nematode

infection tends to be largely Th2 type (IL-4, IL-5, and IL-13 (Craig *et al.* 2007; Meeusen *et al.* 2005b), both these studies also indicated a pro-inflammatory component early on in challenge infection, with Craig et al. demonstrating significant up-regulation of IL-1, IL-6 and TNF-α by Day 5. These cytokine expression data, although in both studies being of local origin (abomasal lymph node and abomasal mucosa), do indicate the possibility that an APPR to infection with *T.circumcincta* may contribute to host control of infection.

The work described here aimed to determine whether there is a significant local APPR to infection with *T.circumcincta*, which could be of potential importance in innate immunity to infection. Observations included determining the levels of selected APP in lymph draining the infection site (the abomasum) as well as determining the transcript levels of these APPs in the abomasal mucosal epithelium.

## *4.2 Results*

### 4.2.1 Acute phase proteins in lymph

Analyses in Chapter 3 suggested that the levels of two of the three major ruminant acute phase proteins (SAA and haptoglobin) in lymph changed during *T. circumcincta* infection, although this change was not significant between any two measured time-points due to between animal variation. SAA and Haptoglobin decreased throughout the experimental infection period, with the difference between Day 0 and Day 21 being the most substantial. Figure 4.1 below shows data from one animal which clearly demonstrates this trend.

In order to confirm this trend and to further quantify the kinetics of the acute phase proteins in lymph from cannulated, infected animals, levels of the acute phase proteins were analysed by ELISA or biochemical assay in animals undergoing a primary infection (Figure 4.2). This data is shown in Figures 4.2 (A), (B) and (C).

SAA levels in lymph (Figure 4.2(A)), decreased throughout the experimental infection period, from a high of 48µg/ml at day 0, to 0.2µg/ml at day 21 post infection. Repeated measures analysis showed the change over time in the primary infection group was significant (Repeated measures, non-parametric, Friedmans test, and Dunn's multiple comparison post test shows that 0 vs 21 $p<0.05$). Pre-infection levels of SAA, as measured 24 and 48 hours prior to challenge, were similar to those observed on the day of challenge (47µg/ml and 52µg/ml respectively). (Pre-infection data is not included in Figure 4.2)

In contrast to SAA, haptoglobin levels (Figure 4.2 (B)) in the primary infection group did not show a statistically significant change over time. However, mean levels were lower at day 21 (0.1mg/ml) compared to day 0 (0.43mg/ml) post infection. Interestingly, pre-infection levels were of a similar magnitude to those observed at day 0. Haptoglobin was measured for 7 days prior to challenge (from the day of cannulation surgery). The highest level was noted 5 days pre-infection (0.78mg/ml). (Pre-infection data is not included in Figure 4.2).

AGP showed no statistically significant change over the experimental infection period, although mean levels were lower at day 21 (155µg/ml), compared to day 0 248µg/ml).

**Figure 4.1: Magnified 2-D gel images focusing on Haptoglobin (A) and SAA (B) spots on a representative 2-D gel of lymph from a naïve sheep challenged with 50,000 *Teladorsagia circumcincta* L3 larvae at Day 0.**

**The left hand images show the spot densities at Day 0 and the right hand images show the respective spots at Day 21 following challenge. In the top right of each image is a bar chart showing the mean normalised spot volume data (mean +SEM, n=5) from gel analysis at Day 0 and Day 21. The change in neither protein is statistically significant, either by repeated measures ANOVA (when day 11 data included, or by paired t-test when just the two time points compared).**

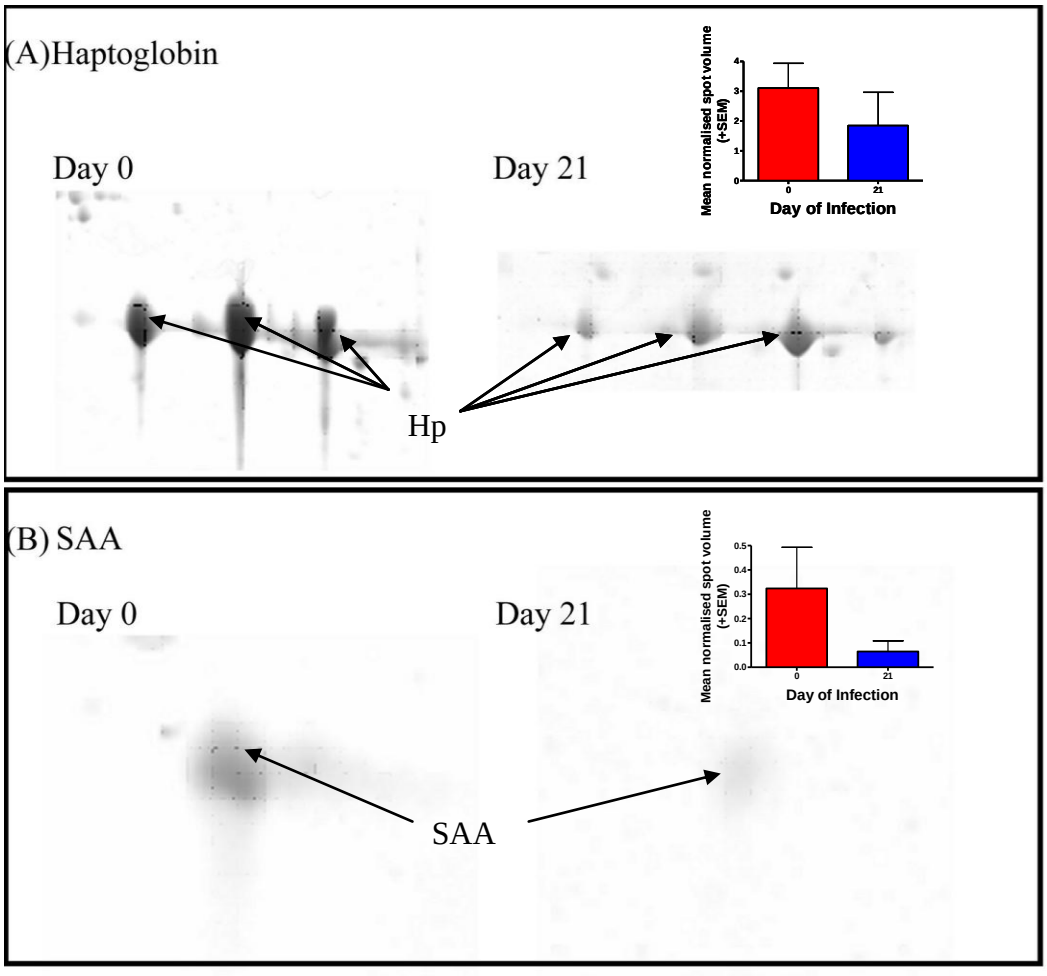
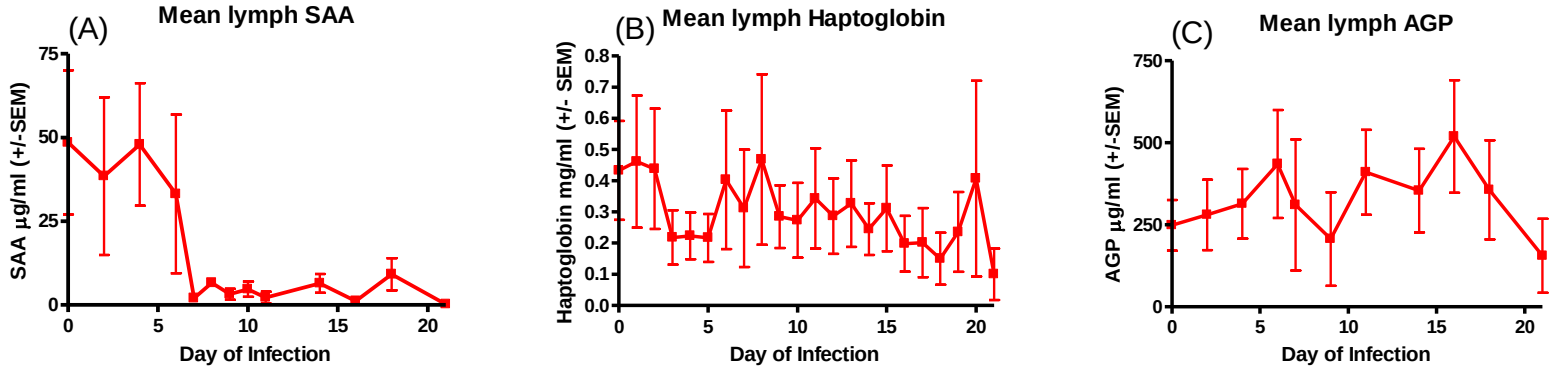


**Figure 4.2: The levels of the acute phase proteins, as measured by ELISA (SAA), biochemical assay (Hp) or immunodiffusion assay (AGP), detectable in gastric lymph following challenge with 50,000 L3 *Teladorsagia circumcincta* larvae.**

**SAA (A) decreased in the primary infection group from a high at day 0 to virtually zero by day 21 ($p<0.05$). Haptoglobin (B) levels were also reduced by day 21 compared to day 0, although this was not statistically significant. AGP (C) showed no significant change over the experimental infection period.**



### 4.2.2 Systemic acute phase protein response to *T.circumcincta*

The systemic response to infection was investigated by measuring the levels of SAA, haptoglobin and AGP in blood. Assays carried out were an ELISA, a biochemical and an immunodiffusion assay respectively for each of the three proteins of interest. The blood used for these experiments was from animals which had not had surgery.

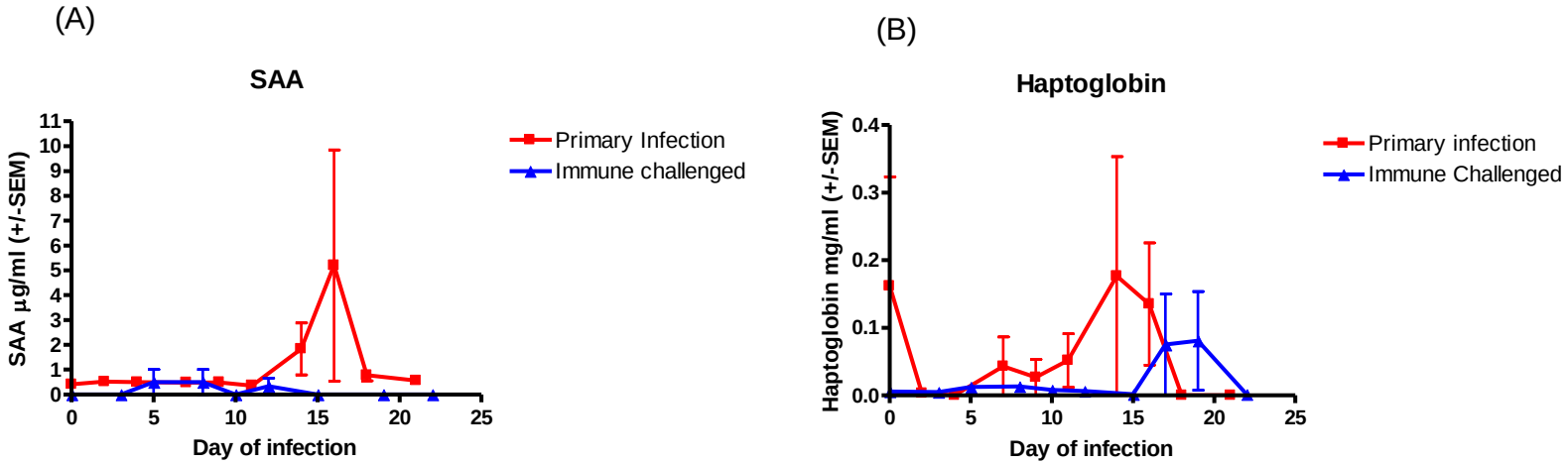
The results for SAA and haptoglobin are shown in Figure 4.3 (A) and (B). There was no significant change over time (as measured by repeated-measures ANOVA) in either of the proteins for either infection group. Statistical comparison between the two infection types (primary versus immune) was unfortunately not possible due to samples being obtained on different days in each group. However, there is a trend in the primary infection group for the levels to be higher around Day 15 of infection, compared to immune challenged animals. This is true for both haptoglobin and SAA. However, it can be seen from the size of the standard error bar that this change is largely due to an unusually high level in one animal at this time point.

AGP levels have not been shown graphically as they were consistently below the detection limit of the assay (<50µg/ml) for both the primary and immune challenged groups.

It is worth noting that the levels of systemic acute phase proteins observed in these experiments fell within the range expected in a healthy, non-diseased individual (Laboratory normal reference ranges: SAA – 0-38 µg/ml, Hp - <0.2mg/ml, AGP – 30-110µg/ml, courtesy of David Eckersall, GUVS). The lack of systemic response was confirmed by real-time PCR (data not shown) using cDNA from liver and the same primers as used for abomasal tissue.

**Figure 4.3 Systemic acute phase protein levels**

**The systemic acute phase response to infection with *T.circumcincta* as detected in blood. SAA levels (A) shown were measured by ELISA. Haptoglobin levels (B) were measured by biochemical assay. For each point shown, n=6. Primary infection (red) are naïve animals undergoing a primary challenge with *T.circumcincta* on Day 0. Immune challenged animals (blue) are previously infected and then infection cleared with anthelmintic 7 days prior to challenge with *T.circumcincta* on Day 0.**



### 4.2.3 Local expression of acute phase proteins

In order to detect local expression of APP, specific primers were designed for each of the three acute phase proteins of interest from available sequence information (see Table 2.4 in Materials and Methods). Each primer pair was used to amplify cDNA from abomasal fold sections. The resultant PCR product, following purification, was sequenced and the sequence obtained identified by means of BLAST searching, using the NCBI nucleotide BLAST search function (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and aligned with its closest match (Figure 4.4 (A), (B), (C)). In this way, it was confirmed that all three of the acute phase proteins were expressed in abomasal tissues.

**Figure 4.4: PCR product sequence alignment and % nucleotide identity with closest BLAST match (Accession number) Region of sequence concensus between forward and reverse reads of sequenced PCR products (Query sequence), aligned with its closest BLAST match (Subject sequence), for SAA (A), Haptoglobin (B) and AGP (C).**

(A) SAA (FJ492830.1) *Bos taurus* SAA3, mRNA; 91%

```
Query  1    CACGCCCGCGGAAACTATGACGCTGCCCAAAGGGGGTCC-AGGGGTGTCTGGGCTGCTGA  59
            |||||||| |||||||||||||||||| || |||| ||  |||||| |||||||||| |
Sbjct  109  CACGCCCGTGGAAACTATGACGCTGCCCGAA-GGGGACCTGGGGGTGCCTGGGCTGCTAA  167

Query  60   AGTGATCAGTAACGGCAGAGAGGCTCTTCAGGGAATCACAGACCCTCTGTTCAAGGGTAT  119
            ||||||||||||| ||||||| || ||||||||||||||||||||| ||||||||
Sbjct  168  AGTGATCAGTAACGCCAGAGAGACTATTCAGGGAATCACAGACCCTCTGTTTAAGGGTAT  227

Query  120  GACCAGGGACCAGGTACGGGAGGATTC  146
            ||||||||||||||||||||||||||
Sbjct  228  GACCAGGGACCAGGTACGGGAGGATTC  254
```

(B) Haptoglobin (NM001040470.1) *Bos taurus* Haptoglobin, mRNA; 98%

```
Query  1    AGGTGGAGAAGGTGGTTCTCCAACCTGACCACTCCAAGGTAGACATTGGGCTCATCAAAC  60
            |||||||||||||||||||| ||||||||||||||||||||||||||||||||||||||
Sbjct  711  AGGTGGAGAAGGTGGTTCTCCACCCTGACCACTCCAAGGTAGACATTGGGCTCATCAAAC  770

Query  61   TCAGACAGAAGGTACCCGTCAATGACAAAGTAATGCCCATCTGCCTACCT  110
            ||||||||||||||| ||||||||||||||||||||||||||||||||
Sbjct  771  TCAGACAGAAGGTACCTGTCAATGACAAAGTAATGCCCATCTGCCTACCT  820
```

(C) AGP (EU127316.1) *Carpa hircus* alpha-1 acid glycoprotein, mRNA; 98%

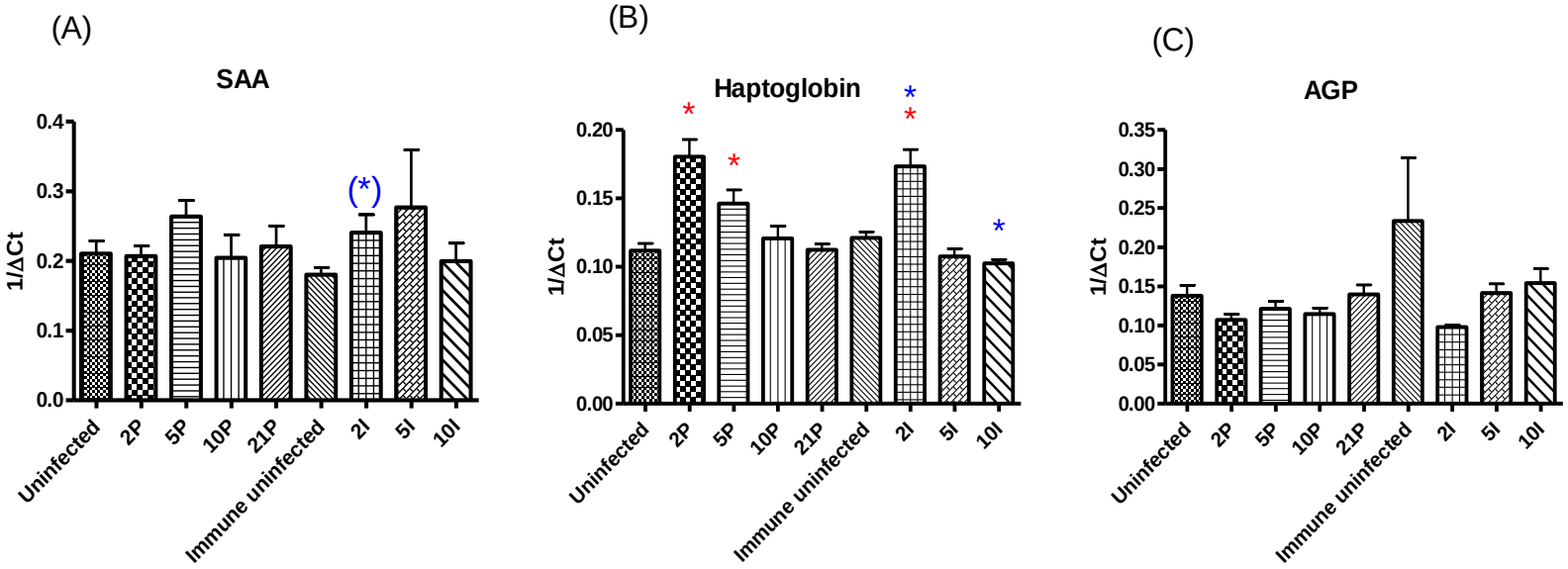
```
Query  57   GCTTTCCGAAACCCTGAGTACAATGAGTCGGCTAGAGCAATCCAGGCGGCTTTCTTTTAC  116
            |||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  172  GCTTTCCGAAACGCTGAGTACAATGAGTCGGCTAGAGCAATCCAGGCGGCTTTCTTTTAC  231

Query  117  TTTGAGCCCAGGCA  130
            ||||||||||||||
Sbjct  232  TTTGAGCCCAGGCA  245
```

### 4.2.4 Real-time (Quantitative) PCR

Following the indication that there may be an acute phase protein response occurring within the abomasal tissue itself, TaqMan probes® were designed for use in a real-time PCR assay. The optimization process for this assay is described in more detail in Materials and Methods, section 2.13.8.

Results of real-time analysis are shown in Figure 4.5. The procedures used to analyse real-time data are described in detail in Materials and Methods, section 2.13.9. Both SAA (Figure 4.5 (A)) and haptoglobin (Figure 4.5 (B)) expression increased early on following infection, compared to uninfected controls. SAA showed maximum expression at day 5 post-infection in both the primary and immune challenged groups, although this change was not statistically significant. Haptoglobin expression was highest at day 2 post-infection in both the primary and immune challenged groups. Expression at this time was significantly higher than in uninfected controls. Haptoglobin expression remained significantly higher at day 5 post-infection in animals undergoing a primary infection, before dropping off to pre-infection levels. The fall in haptoglobin expression in immune animals after the day 2 peak was more rapid, with levels actually being lower than pre-infection levels by day 10 post-infection. In contrast, AGP expression (Figure 4.5 (C)) by abomasal tissue did not change significantly with *T.circumcincta* infection.

**Figure 4.5: Real-time PCR data. Expression of acute phase proteins in abomasal tissue following challenge with *T.circumcincta*. The bar charts show the mean and SEM of ΔCt for each of the time points in infection for SAA (A), Haptoglobin (B) and AGP (C). For purposes of visual display, this has been shown as 1/ΔCt, so that a higher peak represents relatively higher expression. For each infection group, n=6 and four experimental replicates were carried out for every sample. Uninfected refers to worm naïve abomasal tissue and 2P-21P refers to a particular day of a primary infection. Immune uninfected refers to animals previously trickle infected to induce immunity with this infection cleared 7 days prior to sampling and/or challenge. 2I-10I refers to these immune animals at various time points of challenge infection. Statistical analysis was by 2 sample, unpaired, 2 sided t-tests. The red asterisk indicates that this group is significantly different ($p<0.05$) from uninfected abomasum. The blue asterisk indicates that this group is significantly different from immune uninfected ($p<0.05$) abomasum. Brackets around the asterisk indicate that, although $p<0.05$, this point is not significant when the significant p value is adjusted to control for the false discovery rate ($p<0.013$).**



## *4.3 Discussion*

Proteomic analyses (Chapter 3) indicated that SAA and haptoglobin levels decreased in lymph from day 0 compared to day 21 during the infection period (Figure 4.1). Here, the kinetics of the APPR was monitored in more detail by determining SAA, haptoglobin and AGP levels in lymph using ELISA/biochemical assay. The ELISA/biochemical assay (Figure 4.2) data confirmed that SAA and haptoglobin levels tended to decrease in lymph over the infection period. qPCR was used to monitor expression of these proteins in abomasal tissues. qPCR (Figure 4.5) data showed a significant increase in haptoglobin expression early (day 2) in the infection period and a trend for increased SAA at day 5. Neither of these trends were evident in peripheral blood from a group of *T.circumcincta* infected (but non-cannulated) sheep.

APPR has been shown in calves given an experimental challenge with the bovine lungworm *Dictyocaulus viviparus* (Ganheim *et al.* 2004), an infection where there is a larval tissue migratory phase. Calves were exposed to three different dose regimes, all of which induced elevated levels of serum haptoglobin, SAA and fibrinogen, although there was considerable variation both between and within experiments. Previously, Stadnyk et al. (Stadnyk *et al.* 1990) were unable to demonstrate a systemic acute phase response during nematode infection which was confined solely to the gastrointestinal tract. The lack of a systemic acute phase response also coincides, to an extent, with the findings of Conner et al. (Conner *et al.* 1989), who, although recording an increase in haptoglobin in under 50% of animals infected with *Ostertagia ostertagi* (a nematode closely related to *T.circumcincta* in both life-cycle and pathology), were not confident that this inconsistent rise was due to the parasite and conceded that in fact it may have been due to concurrent, unrelated infection. Here, analysis of blood samples following challenge with *T.circumcincta* showed no evidence of a systemic APPR. This is in agreement with the findings of others who have looked at the systemic response to gastrointestinal nematode infection (Conner *et al.* 1989; Stadnyk *et al.* 1990).

The acute phase response is a series of physiologic reactions initiated early in inflammation (Baumann & Gauldie 1994). Inflammatory mediators, such as the cytokines IL-1 and IL-6 are released which induce production of acute phase proteins in the liver and, perhaps, locally at the site of tissues damage. Gene expression of SAA, haptoglobin and AGP were confirmed in abomasal fold samples. The veracity of the SAA, haptoglobin and AGP PCR products was confirmed by sequencing and BLAST analysis followed by sequence alignment (Figure 4.4). The closest SAA and haptoglobin homologues were in *Bos taurus* (Figures 4.4(A) and (B) respectively) and the closest AGP homologue was in *Carpa hircus* (Figure 4.4 (C)). The percent identities (91%, 84% and 98% respectively), confirmed the products. This shows that these acute phase proteins are produced locally and hence raises the possibility that local production of these proteins may be influenced by infection. This would indicate a potential role of the acute phase proteins in either the pathology of the disease or in innate immunity to infection.

Notably, the increased expression level of haptoglobin transcripts at day 2 in both infection groups, and the increased SAA in both groups by day 5 post-infection match in with the cytokine data of Craig et al. (Craig *et al.* 2007), who found that the highest levels of IL-1, IL-6, and TNF-α in abomasal lymph node samples occurred at the earliest time point sampled following infection (day 5) with *T.circumcincta*. This is suggestive that local transcription in the abomasum is stimulated by these cytokines, which is consistent with the findings of others for the role of these cytokines in transcription of the acute phase proteins.

Local expression of haptoglobin may simply be required to bind haemoglobin released from damaged red blood cells. The up-regulation of haptoglobin at the earliest stage of infection may be important due to its property as a protease inhibitor (Pagano *et al.* 1982; Snellman & Sylven 1967) which may allow it to neutralize the enzymatic activity of proteases in larval *T. circumcincta* excretions/secretions. Cathepsin F has been shown to be the most abundant molecule in *T.circumcincta* excretory/secretory products from worms harvested at this time (days 1, 3, 5, 6 and 9 post infection) (Redmond *et al.* 2006; Smith *et al.* 2009). Up-regulation of

haptoglobin may therefore be an innate response designed to inhibit invasion of the mucosa by the immature worms, or to compromise their feeding ability. Alternatively, haptoglobin up-regulation at early time points following infection may stimulate Prostaglandin E release (Baseler & Burrell 1983), leading to greater permeability of blood vessels and allowing the cells and molecules of the immune system greater access to where they are required.

SAA up-regulation at early time points may be solely a consequence of pathology, perhaps with the function of removing cholesterol from dead cells at the site of inflammation (Lindhorst *et al.* 1997). However, it may also have a role in immunity through its ability to attract cells of the immune system (Badolato *et al.* 2000; Xu *et al.* 1995) or in the up-regulation of the membrane bound intestinal mucin gene MUC3 (Larson *et al.* 2003).

It is likely that the APPR in lymph was due to the surgical procedure, surgery being known to induce an APPR (Cray *et al.* 2009; Serin & Ulutas 2010). This is supported by the high levels of haptoglobin and SAA measured in the pre-infection period following surgical cannualtion of the lymph duct. A very rapid surgery induced APPR would be evident in the systemic circulation and this was detectable in lymph, swamping any local APPR. Here local APPR was confirmed by the PCR analyses. The impact of surgery should be controlled for in future by including surgical control animals (not infected with *T.circumcincta*) alongside the infected cannulated animals for the whole duration of the experiment.

In summary, haptoglobin, SAA and AGP were all found to be expressed in ovine abomasal tissues both prior to and following infection with the nematode *T.circumcincta*. Haptoglobin expression was altered significantly by infection, being highest at Day 2 post-challenge in both immune and primary infection models. SAA expression was also up-regulated, especially in the immune group, being highest on Day 5 post-challenge. These data suggest that the acute phase proteins haptoglobin and SAA may have a role in innate immunity or in local immunomodulation to

infection with *T.circumcincta*. Further investigation into the actual role of these proteins in this infection system would be of interest.

# 5 Chapter 5: Gelsolin

## *5.1 Introduction*

The analysis of the gastric lymph proteome showed that, amongst other changes, the amount of gelsolin appeared to decrease significantly in animals undergoing a primary infection with *T. circumcincta* compared to naïve controls. The purpose of this chapter is to verify this finding and to examine the effect of *T. circumcincta* infection on gelsolin expression in more detail.

Gelsolin is the most abundant and the most potent actin depolymerising protein found in mammals (Sun *et al.* 1999b; Vandekerckhove *et al.* 1990). Actin is a ubiquitous cytoskeletal protein essential to many cell functions, both within the cell membrane and in the intracellular environment. Gelsolin occurs in two forms; plasma and cytoplasmic, which attack the non-covalent bonds between actin molecules within a filament thus splitting the filament. It also has a "capping" function which ensures that the actin filaments are unable to reattach to each other and prevents elongation from the capped ends where the gelsolin is attached (Sun *et al.* 1999b).

Circulating gelsolin is important in controlling blood viscosity by scavenging free actin released from dead or damaged cells. Without gelsolin, the blood would become over viscous and fatal circulatory effects could result following tissue trauma. This has been demonstrated in experimentally induced models of oxidative lung damage, sepsis and shock (Christofidou-Solomidou *et al.* 2002; Jordan *et al.* 2007). Christofidou-Solomidou et al. (2002) showed depleted plasma gelsolin following major trauma and found an inverse correlation between gelsolin levels and the subsequent development of respiratory distress. Similarly, mesenteric lymph levels of gelsolin were depleted following hemorrhagic shock (Jordan *et al.* 2007). Gelsolin has also been shown to decrease in plasma of post-surgery patients (Bucki *et al.* 2008; Lee *et al.* 2006). It is thought the reduced levels of plasma gelsolin in the above examples are due to the binding of gelsolin with actin and the subsequent removal of this complex from the circulation.

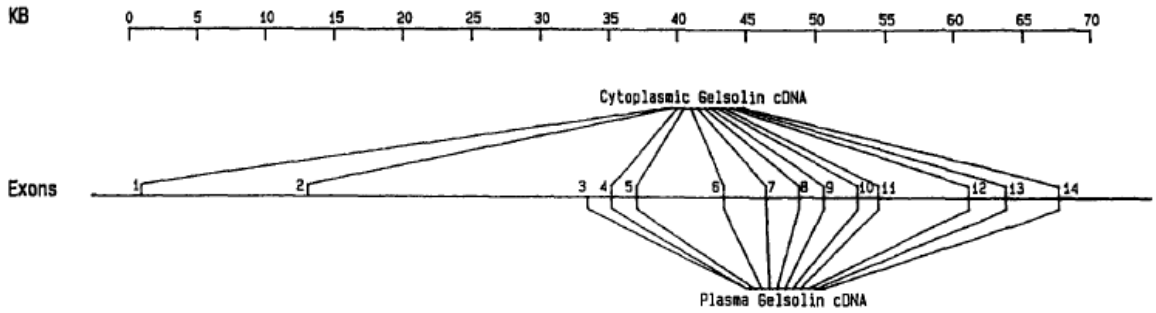
Cytoplasmic gelsolin is thought to be important in actin regulation and re-modelling during cell-division, and in growing or apoptotic cells (Sun *et al.* 1999b). Of potential relevance to the abomasal environment undergoing parasite challenge, it has been shown that actin acts as a barrier to exocytosis in airway goblet cells by forming a distinct sheet between the secretory granules within the cell and their docking sites on the plasma membrane (Ehre *et al*. 2005). When the goblet cells were stimulated with an agonist, the actin barrier was re-modelled and in this way actin was shown to be a negative regulator of mucin secretion in airway goblet cells. Gelsolin has an important role in the reorganisation of actin and so may be involved in this process in the gastrointestinal tract. This has potential interest given the role of mucus in nematode infection (Miller & Huntley 1982).

The importance of gelsolin in this experimental model and indeed in gastric nematode disease is unclear. It may be that the reduction in gelsolin observed in lymph was due to a reduction in plasma gelsolin resulting from actin scavenging following the tissue damage associated with *T. circumcincta* larval damage to the abomasal surface or indeed from surgery. The loss of differentiation of the cells within the parasitized abomasal mucosa alternatively may be the cause of local changes in gelsolin levels in lymph. This raises the question of whether the changes observed in lymph were a reflection of local conditions or merely a reflection of systemic changes in the levels of this protein. Changes in local gelsolin levels may be crucially important in affecting the mucus barrier lining the mucosa, whether that be through altered mucus release from goblet cells due to remodelling of actin barriers, or alteration in mucus viscosity.

Gelsolin is a rather unusual protein being found in both a cytoplasmic form and a secreted plasma form (Bucki *et al.* 2008). The potential roles of abomasally expressed gelsolin could be further elucidated by distinguishing between cytoplasmic and plasma gelsolin. Kwiatkowski et al. (Kwiatkowski *et al.* 1988), have shown in humans that both forms are transcribed from a single gene, using distinct transcriptional initiation sites resulting in two identical proteins that vary only in their 5' end (N terminus). The plasma form of the protein has been described as

having an additional 25 amino acids at the N terminus (Yin *et al.* 1984). Shown below (Figure 5.1) is the genomic organisation of gelsolin transcription as proposed by Kwiatkowski et al (Kwiatkowski *et al.* 1988).

**Figure 5.1: Map of gelsolin gene. DNA size markers are shown at top in kilobases. This figure is adapted from (Kwiatkowski *et al.* 1988).**



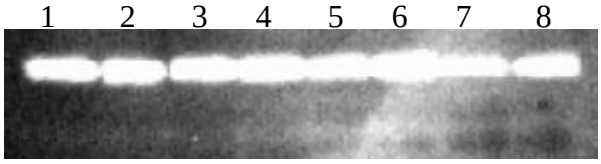

The aim of this chapter is to investigate the effect of *T.circumcincta* infection on gelsolin protein levels, and also on local gelsolin gene transcription. Since the gelsolin gene has not yet been described in sheep, this has required the identification of ovine gelsolin splice variants.

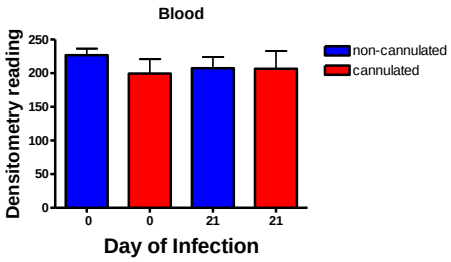
## *5.2 Results*

### 5.2.1 Circulating levels of gelsolin in blood plasma

It has been shown by others that circulating gelsolin levels fall following tissue trauma. Here, it was possible that gelsolin levels could be affected by surgery. This was addressed by comparing circulating gelsolin levels in blood plasma from surgically cannulated and non-cannulated animals which received a primary challenge infection. Gelsolin was detected using a semi-quantitative immunoblot technique (Figure 5.2). This analysis indicated that circulating gelsolin levels in cannulated and non-cannulated sheep were similar and were unaffected by the time after cannulation.

**Figure 5.2: Western blot showing blood plasma from cannulated and non-cannulated sheep following a primary challenge with *T.circumcincta*. This blot was carried out using anti-human monoclonal gelsolin antibody and a chemiluminescent substrate (see Materials and Methods). Equal volumes (1µl) of blood plasma were run in each lane. The figure shows gelsolin levels in blood from cannulated and non-cannulated sheep (two of each) at days 0 and 21 post-infection (these blots are representative of blots run for four animals in each group (cannulated and non-cannulated) The combined mean densitometry data for each group is shown in the bar chart + SEM (n=4). There was no significant difference between the groups (p=0.577) or between time points (p=0.673) (General Linear Model Repeated Measures, with one paired (time) and one independent factor (group); IBM SPSS Statistics 18.)**




**1: Sheep 1394, day 0, non-cannulated**

**2: Sheep 1063P, day 0, cannulated**

**3: Sheep 1394, day 21, non-cannulated**

**4: Sheep 1063P, day 21, cannulated**

**5: Sheep 1431, day 0, non-cannulated**

**6: Sheep 955P, day 0, cannulated**

**7: Sheep 1431, day 21, non-cannulated**

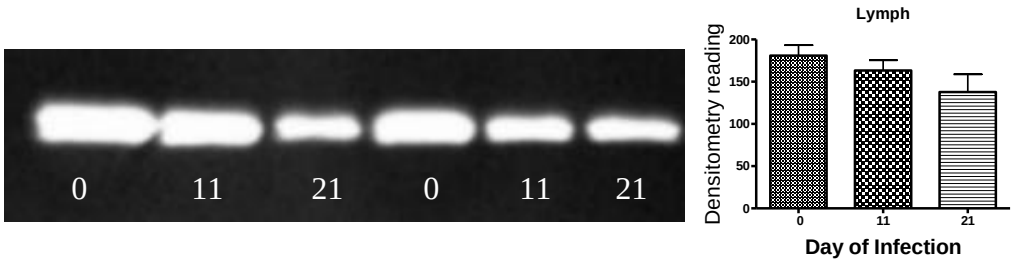
**8: Sheep 955P, day 21, cannulated**

### 5.2.2 Gelsolin levels in abomasal lymph

Work described in chapter 3 (summarized in Figure 3.7) suggested that gelsolin levels decreased in lymph of sheep undergoing a primary infection with *T.circumcincta*. Here, confirmation of this observation was sought by the semi-quantitative detection of gelsolin in lymph from animals given a *T. circumcincta* primary infection. Gelsolin was detected by probing Western blots of lymph proteins with anti-gelsolin antibody, using days 0, 11 and 21 post-infection lymph samples (n=5) (Figure 5.3). The signal strength on the blot clearly diminished in all animals as infection progressed ($p<0.01$).

**Figure 5.3: (A) Western blot of lymph at days 0, 11 and 21 days following a primary challenge with *Teladorsagia circumcincta* from two different animals. The blot was developed using anti-human monoclonal gelsolin antibody and chemiluminescent substrate (see Materials and Methods), and equal volumes of lymph were added to each lane. A clear decrease in gelsolin levels is evident between Days 0 and 21. (These blots are representative of blots from five animals.) (B) The combined mean densitometry data for each time point is show in the bar chart +SEM (n=5). Lymph gelsolin levels were significantly lower at Day 21 compared to Day 0 ($p<0.01$, repeated measures ANOVA).**

(A) (B)

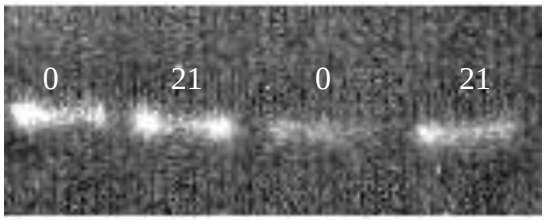



The gelsolin levels in lymph are likely to be a reflection of the systemic levels found in blood but influenced by the local tissue environment. To clarify this, blood levels from cannulated sheep were estimated using the same western blot methodology on

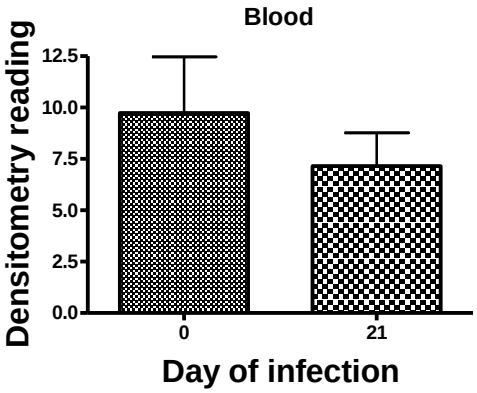
Day 0 and Day 21 post infection (Figure 5.4). No significant decrease in blood gelsolin levels was evident when day 0 and day 21 were compared in the six animals.

**Figure 5.4: (A)Western blot showing blood plasma from two cannulated sheep following a primary challenge with *T.circumcincta*. This blot was carried out using anti-human monoclonal gelsolin antibody and a chemiluminescent substrate (see Materials and Methods). Equal volumes (0.05µl) of blood plasma were run in each lane. The blot shows gelsolin levels in blood from two cannulated animals at Day 0 and Day 21 post infection. (This blot is representative of the blots from six animals). (B)The combined mean densitometry data for each time point is shown in the bar chart + SEM (n=6). There was no significant difference between the days (paired t-test, p=0.4).**

(A)




(B)




In order to more accurately investigate the kinetics of systemic gelsolin following surgery and/or infection, a more sensitive method of investigation was required. This was addressed by attempting to develop a gelsolin Enzyme Linked Immunosorbent Assay (ELISA). Because no anti-ovine gelsolin antibodies were available, assay development used a polyclonal mouse anti-human gelsolin antibody. This antibody recognized a protein at ~80 kDa when used to probe immunoblots of lymph consistent with the expected size of the native molecule. However, attempts to develop a quantitative ELISA failed for a variety of reasons (see Discussion) and this approach was discontinued. An alternative and highly sensitive approach was to monitor abomasal gelsolin mRNA transcript abundance using Real-time PCR

(qPCR). To accomplish this, there was a requirement to determine the sequence of ovine gelsolin and the possible impact of splice variants on the specificity and sensitivity of any qPCR assay evaluated.

### 5.2.3 Isolation of the ovine gelsolin coding sequence

Initially, a search was made of the available databases. No ovine sequence was available for the gelsolin gene from the NCBI database at the time of searching. However, the bovine sequence was available on NCBI and this was used to search in the sheep expressed sequence tag (EST) library databases (NCBI/UniGene/ovis aries) for matching ovine sequence. A total of eleven partial sequences were acquired and then aligned using ClustalW. Six of the sequences encoded the 5' end and these fell into three distinct 5' sequences. Figure 5.5 shows an alignment of the derived (Transeq software) amino acid sequences with the amino acid sequence for the human plasma and cytoplasmic variants (using Clustal W software). The alignment shows that ovine variant 1 had the closest homology to the human plasma isoform, and contained a signal peptide (27 amino acids) in the same position as the human plasma form. Ovine variants 2 and 3 most closely resembled the human cytoplasmic isoform and Signal P analysis predicted that these proteins are not secreted and are likely to represent cytoplasmic forms of gelsolin. Ovine variant 2 had an additional 11 amino acids at its 5' end compared to variant 3 and human cytoplasmic gelsolin. Three separate putative gelsolin sequences were constructed, although there was no independent verification of these sequences. For the purpose of this study, these three sequences were named plasma (ovine variant 1), cytoplasmic 1 (ovine variant 2), and cytoplasmic 2 (ovine variant 3), due to their similarities with the published human sequence.

Figure 5.6 shows an alignment of the plasma (ovine variant 1), cytoplasmic 1 (ovine variant 2), and cytoplasmic 2 (ovine variant 3) nucleotide sequences at their 5' ends, created using Clustal W software. In order to verify these predictions, PCR primers were designed to enable the differential amplification of the cytoplasmic and plasma variants as well as confirming their sequence and their expression in abomasal tissue.

PCR reactions using these primers and abomasal cDNA as target did yield bands of the expected size. An image showing the two bands obtained using the cytoplasmic variant specific primers is shown in Figure 5.7. The plasma variant specific primer produced a single band of approximately 114bp as predicted. The identity of the products was confirmed by sequence analysis (Figure 5.8). Multiple reactions were carried out and the products of each of these were cloned and expressed in a similar way in order to give final consensus sequences. Primer sequences and assay conditions are available in Materials and Methods, Table 2.3.

The partial ovine sequences were then BLAST searched and the best matches are shown in the table below (Table 5.1). Alignment of the putative gelsolin sequences (Figure 5.6) with those obtained from sequencing of PCR product (Figure 5.8) shows that the sequences are identical, except for a possible A-G polymorphism at position 143 in the sequenced cytoplasmic 1 (cyto_1) variant (alignment not shown).

**Figure 5.5: Alignment of the amino acid sequences of the two known human isoforms, (plasma/secreted and cytoplasmic, accession numbers P06396-1, P06396-2, respectively), with the three ovine variants detected in the ovine EST database (DY519479, DY504741, EE828632, EE819856, EE812207, DY498593, EE773637, DY493807, EE864233, EE862820, EE858555). Highlighted in yellow is the area of signal peptide in the human plasma form (data from UniProtKB), and also the putative signal peptide in the ovine variant 1 (as predicted by Signal P3.0 server (Bendtsen *et al*. 2004)). The sequence shown corresponds to the first 200 amino acids of the human plasma gelsolin protein (full size: 782AA).**

```
ovine_variant_1             MAAHR--CALLGALVLALGALSQPARPATPGRGAAQARAPQGRVTEARPS 48
ovine_variant_2             ----------------------------------------MENLFCCFPS 10
ovine_variant_3             -------------------------------------------------
human_gelsolin_cytoplasmic  -------------------------------------------------
human_gelsolin_plasma       MAPHRPAPALLCALSLALCALSLPVRAATASRGASQAGAPQGRVPEARPN 50


ovine_variant_1             SMVVEHPEFLKAGKEPGLQIWRVEKFDLVPVPPNLYGDFFTGDAYVILKT 98
ovine_variant_2             SMVVEHPEFLKAGKEPGLQIWRVEKFDLVPVPPNLYGDFFTGDAYVILKT 60
ovine_variant_3             -MVVEHPEFLKAGKEPGLQIWRVEKFDLVPVPPNLYGDFFTGDAYVILKT 49
human_gelsolin_cytoplasmic  -MVVEHPEFLKAGKEPGLQIWRVEKFDLVPVPTNLYGDFFTGDAYVILKT 49
human_gelsolin_plasma       SMVVEHPEFLKAGKEPGLQIWRVEKFDLVPVPTNLYGDFFTGDAYVILKT 100
                             ********************************.*****************


ovine_variant_1             VQLRNGNLQYDLHYWLGNECSQDESGAAAIFTVQLDDHLNGRAVQHREVQ 148
ovine_variant_2             VQLRNGNLQYDLHYWLGNECSQDESGAAAIFTVQLDDHLNGRAVQHREVQ 110
ovine_variant_3             VQLRNGNLQYDLHYWLGNECSQDESGAAAIFTVQLDDHLNGRAVQHREVQ 99
human_gelsolin_cytoplasmic  VQLRNGNLQYDLHYWLGNECSQDESGAAAIFTVQLDDYLNGRAVQHREVQ 99
human_gelsolin_plasma       VQLRNGNLQYDLHYWLGNECSQDESGAAAIFTVQLDDYLNGRAVQHREVQ 150
                            ************************************:************


ovine_variant_1             GFESATFLGYFKSGLKYKKGGVASGFKHVVPNEVVVQRLFQVKGRRVVRA 198
ovine_variant_2             GFESATFLGYFKSGLKYKKGGVASGFKHVVPNEVVVQRLFQVKGRRVVRA 160
ovine_variant_3             GFESATFLGYFKSGLKYKKGGVASGFKHVVPNEVVVQRLFQVKGRRVVRA 149
human_gelsolin_cytoplasmic  GFESATFLGYFKSGLKYKKGGVASGFKHVVPNEVVVQRLFQVKGRRVVRA 149
human_gelsolin_plasma       GFESATFLGYFKSGLKYKKGGVASGFKHVVPNEVVVQRLFQVKGRRVVRA 200
                            ************************************************
```

**Figure 5.6: Three putative gelsolin sequences corresponding to ovine variant 1 (plasma), ovine variant 2 (cytoplasmic 1) and ovine variant 3 (cytoplasmic 2). Areas targeted with primers designed to enable differential amplification of the variants have been highlighted. These primers would be expected to give product sizes of ~114bp, ~335bp, and ~263bp for plasma, cytoplasmic 1 and cytoplasmic 2 respectively.**

```
1_cyto_1        -----------CGCAGCCGCCGTCGCCAGCGCCGCAGCCGCAG-GTGGTACTGGGAACCC 48
2_cyto_2        CAGCTGGACCCCGCAGCCGCCGTCGCCAGCGCCGCAGCCGCAG-GTGGTACTGGGAACCC 59
3_plasma        ---------CCCGGGTCTTCCGCCGCCGTCGCTGCCATGGCTGCGCATCGCTGCGCGCTG 51
                          **   *  *** ****  *** **    ** * *     *** *  *

1_cyto_1        CCCGAGCCGCGCTTCCACAGTGGTGCCGAGCCCTTCCGAGGGAGCCGCCCTCTTCACTCA 108
2_cyto_2        CCCGAGCCGCGCTTCCACAGTGGTGCCGAGCCCTTCCGAGGGAGCCGCCCTCTTCACTCA 119
3_plasma        CTGGGCGCGCTGGTCC----TGGCGCTGGGCGCG-CTGTCGCAGCCGGCCCGGCCGGCCA 106
                *  *   ***   ***    *** ** * ** *  * *  * ***** **    *   **

1_cyto_1        CAGCCCCACGAGTTCTTGCAGATACA-GCGCTAGGAAAAGGGGAGTAATTCAGGTCTAGA 167
2_cyto_2        CAGCCCCACGAG---------------------------------------------- 131
3_plasma        CCCCGGGGCGGGGGGCGGCCCAGGCACGGGCGCCGCAGGGGCGAGTGACCGAGGCGCGG- 165
                *  *    ** *

1_cyto_1        ATGGAAAACCTGTTTTGTTGCTTTCCGAGCAGCATGGTGGTGGAACACCCCGAGTTCCTC 227
2_cyto_2        ------------------------CCGAGCAGCATGGTGGTGGAACACCCCGAGTTCCTC 167
3_plasma        ------------------------CCGAGCAGCATGGTGGTGGAACACCCCGAGTTCCTC 201
                                        **********************************

1_cyto_1        AAGGCAGGGAAGGAGCCTGGCCTGCAGATCTGGCGTGTGGAGAAGTTCGACCTGGTTCCC 287
2_cyto_2        AAGGCAGGGAAGGAGCCTGGCCTGCAGATCTGGCGTGTGGAGAAGTTCGACCTGGTTCCC 227
3_plasma        AAGGCAGGGAAGGAGCCTGGCCTGCAGATCTGGCGTGTGGAGAAGTTCGACCTGGTTCCC 261
                ************************************************************

1_cyto_1        GTGCCCCCCAACCTTTACGGAGACTTCTTCACAGGCGATGCCTATGTCATCCTGAAGACG 347
2_cyto_2        GTGCCCCCCAACCTTTACGGAGACTTCTTCACAGGCGATGCCTATGTCATCCTGAAGACG 287
3_plasma        GTGCCCCCCAACCTTTACGGAGACTTCTTCACAGGCGATGCCTATGTCATCCTGAAGACG 321
                ************************************************************

1_cyto_1        GTGCAGCTGAGGAATGGGAACCTGCAGTATGACCTCCACTACTGGCTGGGCAATGAGTGC 407
2_cyto_2        GTGCAGCTGAGGAATGGGAACCTGCAGTATGACCTCCACTACTGGCTGGGCAATGAGTGC 347
3_plasma        GTGCAGCTGAGGAATGGGAACCTGCAGTATGACCTCCACTACTGGCTGGGCAATGAGTGC 381
                ************************************************************
```

**CCG… "cytoplasmic" variants forward primer region**

**CGA… "plasma" variant forward primer region**

**CGT… "plasma" variant reverse primer region**

**AAG… "cytoplasmic" variants reverse primer**

**Figure 5.7: Agarose gel image of PCR product from plasma and cytoplasmic variant specific primers. Image A shows the single band of PCR product obtained from using the plasma primers. Image B shows two bands of PCR product obtained from a reaction using the cytoplasmic primers. The size of these bands corresponds to the expected size of products based on the sequence in Figure 5.6 (~114 bp (plasma) and ~263bp and ~335bp (cytoplasmic)). These bands were individually excised, cloned and sequenced.**

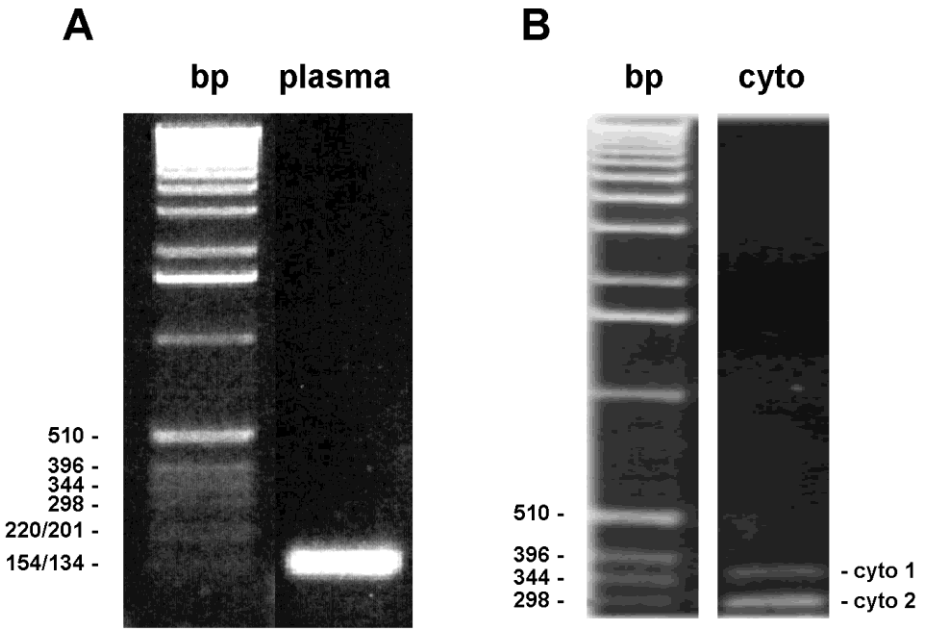


**Figure 5.8: Sequences obtained from PCR using the primers highlighted in Figure 5.6. PCR products were cloned and sequenced.**

```
cyto1           TCCGCAGGTGGTACTGGGAACCCCCCGAGCCGCGCTTCCACAGTGGTGCCGAGCCCTTCC 60
cyto2           TCCGCAGGTGGTACTGGGAACCCCCCGAGCCGCGCTTCCACAGTGGTGCCGAGCCCTTCC 60
plasma          ------------------------------------------------------------


cyto1           GAGGGAGCCGCCCTCTTCACTCACAGCCCCACGAGTTCTTGCAGATACAGCGCTAGGGAA 120
cyto2           GAGGGAGCCGCCCTCTTCACTCACAGCCCCACGAG------------------------- 95
plasma          ------------------------------------------------------------


cyto1           AGGGGAGTAATTCAGGTCTAGAATGGAAAACCTGTTTTGTTGCTTTCCGAGCAGCATGGT 180
cyto2           ----------------------------------------------CCGAGCAGCATGGT 109
plasma          --------------------TGCAGGGGCGAGTGACCGAGGCGCGGCCGAGCAGCATGGT 40
                                                              **************


cyto1           GGTGGAACACCCCGAGTTCCTCAAGGCAGGGAAGGAGCCTGGCCTGCAGATCTGGCGTGT 240
cyto2           GGTGGAACACCCCGAGTTCCTCAAGGCAGGGAAGGAGCCTGGCCTGCAGATCTGGCGTGT 169
plasma          GGTGGAACACCCCGAGTTCCTCAAGGCAGGGAAGGAGCCTGGCCTGCAGATCTGGCGTGT 100
                ************************************************************


cyto1           GGAGAAGTTCGACCTGGTTCCCGTGCCCCCCAACCTTTACGGAGACTTCTTCACAGGCGA 300
cyto2           GGAGAAGTTCGACCTGGTTCCCGTGCCCCCCAACCTTTACGGAGACTTCTTCACAGGCGA 229
plasma          GGAGAAGTTCGACCTA---------------------------------------------- 116
                ***************


cyto1           TGCCTATGTCATCCTGAAGACGGTGCAGCTGAGGA 335
cyto2           TGCCTATGTCATCCTGAAGACGGTGCAGCTGAGGA 264
plasma          -----------------------------------
```

**Table 5.1 : Results of BLAST searching of the partial ovine sequences obtained from sequenced PCR product.**

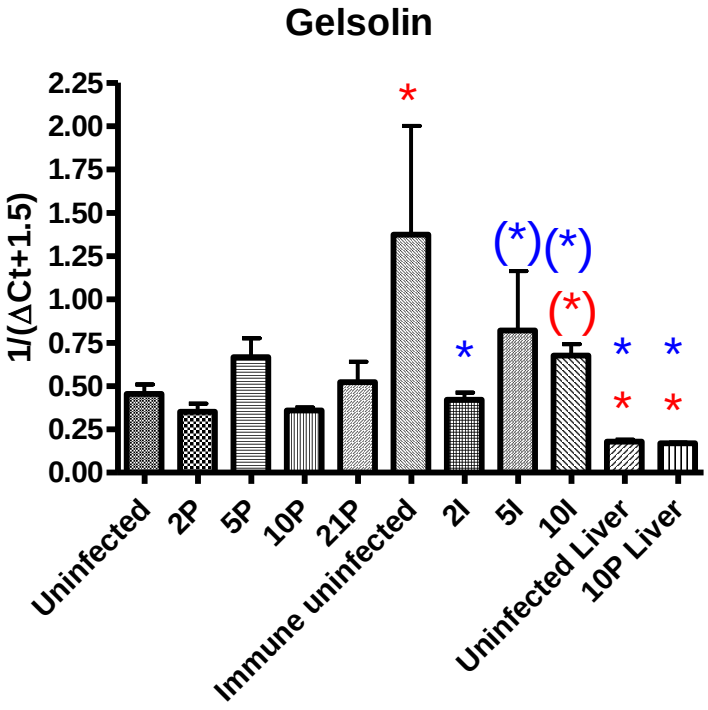
| Ovine variant | Closest BLAST match | Accession number | Query Coverage (%) |
|---|---|---|---|
| Cytoplasmic 1 | Pan troglodytes gelsolin, transcript variant 8 | NM001160857 | 87 |
| Cytoplasmic 2 | Bos taurus gelsolin, transcript variant 2 | NM001034627 | 99 |
| Plasma | Bos taurus gelsolin, transcript variant 1 | NM001113284 | 98 |

### 5.2.4 Abomasal expression of gelsolin by qPCR

Quantitative kinetics of gelsolin mRNA expression within the local environment of the abomasum during infection with *T.circumcincta* was determined using qPCR. A primer and probe set was selected from an area of sequence common to all three variants (Materials and Methods, Table 2.4) and gave a product size of 76 bp. Primer products were sequenced to confirm that the correct sequence was amplified. The results of the qPCR analyses are summarized in Figure 5.9.

In the primary infection group, levels altered little over the infection period and no obvious trend was apparent. Gelsolin transcript relative copy number was much greater in immune abomasa compared to uninfected controls. It declined rapidly in the period immediately after infection (day 2) before increasing again by day 5. This change was statistically significant in the immune uninfected samples versus the day 2 post infection samples ($p<0.013$).

**Figure 5.9: Relative expression of gelsolin in abomasal tissue and liver. The bar charts show the mean and SEM of ΔCt. For purposes of visual display 1 /(ΔCt+1.5) has been shown, hence the higher the bar, the higher the expression relative to the housekeeping gene (sheep ATPase). For each infection group, n=6 (except 10P liver, when n=4) and four experimental replicates were carried out for every sample. Uninfected refers to worm naïve abomasal tissue and 2P-21P refers to a particular day of a primary infection. 2I-10I refers to immune animals at various time points of challenge infection. Uninfected liver is liver from worm naïve animals, and 10P liver refers to liver from animals on Day 10 following a primary infection. Further information of the analysis of the data is available in Material and Methods (section 2.13.9). The red asterisk indicates that this group is significantly different ($p<0.05$) from Uninfected abomasum. The blue asterisk indicates that this group is significantly different from Immune Uninfected ($p<0.05$) abomasum. Brackets around the asterisk indicate that, although $p<0.05$, this point is not significant when the significant p value is adjusted to control for the false discovery rate ($p<0.013$).**



## 5.3 Discussion

Gelsolin is a multifunctional actin-binding protein, which is highly conserved across mammalian species (Bucki *et al.* 2008). It is present in a wide range of cells and exists in extracellular (83kDa) and cytoplasmic (81kDa) forms that are generated from the same gene by initiation of transcription at alternative sites and selective processing of RNA (Bucki *et al.* 2008; Kwiatkowski *et al.* 1988). In chapter 3, gelsolin levels in lymph draining the abomasum during a primary infection with *T.circumcincta* were found to decrease with time. In this chapter these results were further verified by means of western blot analysis. Blood plasma gelsolin levels from cannulated and non-cannulated animals were also studied by western blot and no difference was detected between the groups and indeed blood plasma levels showed no evidence of reduction between days 0 and 21, unlike the lymph levels from the same animals.

Local expression studies revealed that three gelsolin variants are transcribed in the ovine abomasum. A primer and probe set designed to pick up all three variants was used in a qPCR study to evaluate the effect of *T.circumcincta* on abomasal gelsolin expression. This showed gelsolin expression to be significantly higher in immune uninfected abomasum compared to uninfected naive tissue. Gelsolin expression was significantly lower on day 2 post infection in immune animals compared to unchallenged immune animals.

Gelsolin levels in blood plasma were compared from surgically cannulated and non-cannulated animals (Figure 5.2). This was to determine whether the reduction observed in lymph over the 21 day infection period was due to a fall in systemic gelsolin levels post-surgery. Gelsolin levels have been shown to decrease in humans following surgery (Bucki *et al.* 2008; Lee *et al.* 2006). The results did not support this hypothesis as no discernible difference could be detected between blood levels in cannulated compared to non-cannulated animals. In addition, the blood plasma levels did not appear to show the same trend (decrease) observed in lymph (Figure 5.4), suggesting that this effect was indeed due to local factors.

In order to improve on the semi-quantitative technique of western blotting, an ELISA protocol was investigated, but was unfortunately unsuccessful. The failure of the ELISA may have been due to a number of factors. Anti-human antibodies were used as no anti-ovine (or any other ruminant species) gelsolin antibodies were available. This may have reduced the sensitivity and specificity of the assay. The prototype ELISA worked better (although still poorly) when the plate was coated with the sample in a capture ELISA format as opposed to a sandwich ELISA. Other published methods to measure gelsolin are based on the biological activity of gelsolin (Cooper *et al.* 1983; Smith *et al.* 1987a) and could be explored in the future.

Several studies measuring changes in gelsolin levels focus on tissue specific production by means of PCR techniques. Putative sequence data obtained from ovine EST databases (Figure 5.5 and 5.6) was confirmed through the design of specific primers, followed by cloning and sequencing of PCR product. This confirmed that three gelsolin variants are transcribed in the ovine abomasum (Figure 5.8). Sequence similarities to the human plasma and cytoplasmic variants (Figure 5.5) led to these variants being putatively designated plasma (1) and cytoplasmic (2). Further investigation of the local effect of *T.circumcincta* was therefore continued by means of qPCR. From the sequences obtained TaqMan® probes were designed which would pick up all three gelsolin variants when used in the qPCR assay. Primers capable of distinguishing between variants would have been interesting but were not pursued at this stage due to difficulty in successful design. This was largely due to the high GC content in the area of interest leading to unacceptably high melting temperatures. Until gelsolin transcription was known to alter, the costs involved in the production of probes and assays with a high potential of failure could unfortunately not be justified.

Although gelsolin levels in lymph in primary infection were found to decrease throughout the 21 day infection period (Chapter 3 and Figure 5.3), this change was not reflected in the local transcription of gelsolin mRNA (Figure 5.9). Transcription of gelsolin did not significantly vary over the 21 day period in primary infection animals and there was no trend evident. This suggests that the reduced lymph levels

observed are most likely due to increased local consumption of gelsolin, as opposed to reduced local production. Increased requirement for gelsolin may occur in the parasitized abomasum due to tissue damage caused by the invading worms and a greater need for actin scavenging. Alternatively circulating gelsolin may affect the mucus viscosity in the abomasum. It has been shown that gelsolin reduces airway mucus viscosity in vitro (Vasconcellos *et al.* 1994a). Circulating gelsolin may gain access to the mucus lining the abomasum by means of "leak-lesions" known to occur in *T.circumcincta* infection (Murray 1969; Murray *et al.* 1971; Simpson 2000).

No significant change in gelsolin levels was observed in the proteomic analysis of lymph draining the abomasum in immune animals undergoing a challenge infection (Chapter 3). Interestingly though, local transcription of gelsolin was significantly higher in unchallenged immune animals, compared to unchallenged naive animals (Figure 5.9). This agrees with the findings of Athanasiadou et al.(Athanasiadou *et al.* 2008) who, by proteomic analysis of mucosal washings from an in vitro organ *T.circumcincta* challenge model, also suggested that gelsolin may be more abundant in immune (challenged and unchallenged), compared to naïve (challenged and unchallenged) ovine abomasal tissue. It is conceivable that the increased gelsolin transcription observed in the current study may reflect the complement of cell types present in the immune abomasum. Macrophages are known to express high levels of gelsolin (Hashimoto *et al.* 1999) and therefore recruitment of macrophages would lead to an increase in local gelsolin mRNA abundance. However, the human protein atlas immunohistochemistry resource (www.proteinatlas.org) (non-peer reviewed results) shows strong immunohistochemical localisation of gelsolin to the glandular epithelium of the stomach (specifically mucoid cells) and so these levels may indeed reflect abomasal epithelial transcription itself.

It is interesting to note that gelsolin levels fell sharply at day 2 post-infection in immune animals, before increasing again. The reason for this reduction in transcription is not known. It is possible that a sudden reduction in secreted gelsolin could lead to thickening of the mucus (Vasconcellos *et al.* 1994a), making it harder for the nematode to penetrate the gastric glands. This hypothesis would be in

agreement with the idea that mucus is crucial for immunity to gastrointestinal parasites (Miller 1987) through trapping or excluding the nematode and preventing habitation of its niche.

In contrast, the levels increase after day 2 and although not statistically significant, do show a trend towards higher levels than in the primary infection tissues. This may be a reflection of the Th-2 bias of the immune response to gastrointestinal nematodes (Artis 2006b; Craig *et al.* 2007). IL-4 has been shown to increase transcription and secretion of gelsolin into airway surface liquid by epithelial cells in vitro (Candiano *et al.* 2005). IL-4 in the abomasal lymph node is significantly upregulated in immune challenged sheep by day 5 post infection, compared to naïve uninfected controls (Craig *et al.* 2007). Gelsolin may then form part of a more complex IL-4 controlled anti-inflammatory response.

Liver was included as a tissue control to try and show that any observed change in the abomasum was a local effect of the worms, as opposed to a local consequence of a systemic effect. It would have been useful to include liver from immune animals, but these samples were not available at the time of carrying out this study. Gelsolin levels in liver did not change in the sample categories measured. It is interesting to note that liver has significantly less gelsolin transcription relative to abomasum. This suggests that gelsolin may have a greater role in the abomasum compared to liver.

In summary, the work here and in Chapter 3, combined with the information available in the literature, suggests that gelsolin may play an important role in the response to *T.circumcincta* infection in the immune abomasum. The presence of two cytoplasmic forms of gelsolin appears to be novel and would be an interesting area to follow up. Clearly further work would be of interest to look at why gelsolin expression is elevated in the immune abomasum and the reasons for its sudden depletion following challenge. Immunohistochemistry or in situ hybridisation are both techniques which could be used to this end, with an aim of identifying which cell types produce most gelsolin and whether the observed changes reflect a change

in the cell population, or a change in transcription levels in the pre-existing cell types.

Futhermore, since qPCR indicated significantly elevated expression in immune sheep, this justifies further detailed quantification of individual gelsolin variants over the course of infection. Only through a combination of these techniques will the true complexity of gelsolin's role in the host response to *T.circumcincta* infection be unravelled.

# 6 Chapter 6: Tight junctions in the abomasum

## *6.1 Introduction*

The abomasal mucosal epithelium acts a barrier between the non-sterile environment of the abomasal lumen, and the sub-mucosa. The mucosal epithelium is composed of simple columnar epithelium, and between each cell are tight junctions, which control movement and diffusion between the cells. *T.circumcincta* infection is associated with an immediate-type hypersensitivity reaction (Huntley *et al.* 1987). This leads to the formation of a characteristic "leak lesion" phenomenon (Murray *et al.* 1971) whereby the integrity of this mucosal barrier is temporarily altered allowing the passage of substances not normally expected to diffuse over the abomasal mucosa. In parasitised mice, increased intestinal permeability has been attributed at least in part to mast cell degranulation mediated by IL-4 as part of a Th 2 response (McDermott *et al*. 2003). Specifically, the mast cell proteinase, mouse mast cell proteinase-1 (MMCP-1), has been linked to the intestinal pathology associated with *Trichinella spiralis* infection (Lawrence *et al.* 2004).

Increased permeability during *T.circumcincta* infection leads to the passage of pepsinogen from the abomasal lumen to the sub-mucosa, and then on to the lymph and systemic circulation (McKellar 1993; Simpson 2000; Smith *et al.* 1983a; Smith *et al.* 1983b). The rise in pepsinogen mirrors the abundance of mucosal mast cells (Smith *et al*. 1984) and it has been proposed that the "leak lesion" is a consequence of mast cell degranulation in the abomasal mucosa, in turn, allowing potential immune effector molecules access to the mucous layer (Yakoob *et al.* 1983). The mast cell proteinase, sheep mast cell proteinase (SMCP), can be detected in lymph from immune animals undergoing a challenge infection between 1 and 4 days post challenge (Huntley *et al.* 1987), the timing of which coincides with the presence of the leak lesion in immune animals (Smith *et al.* 1983a).

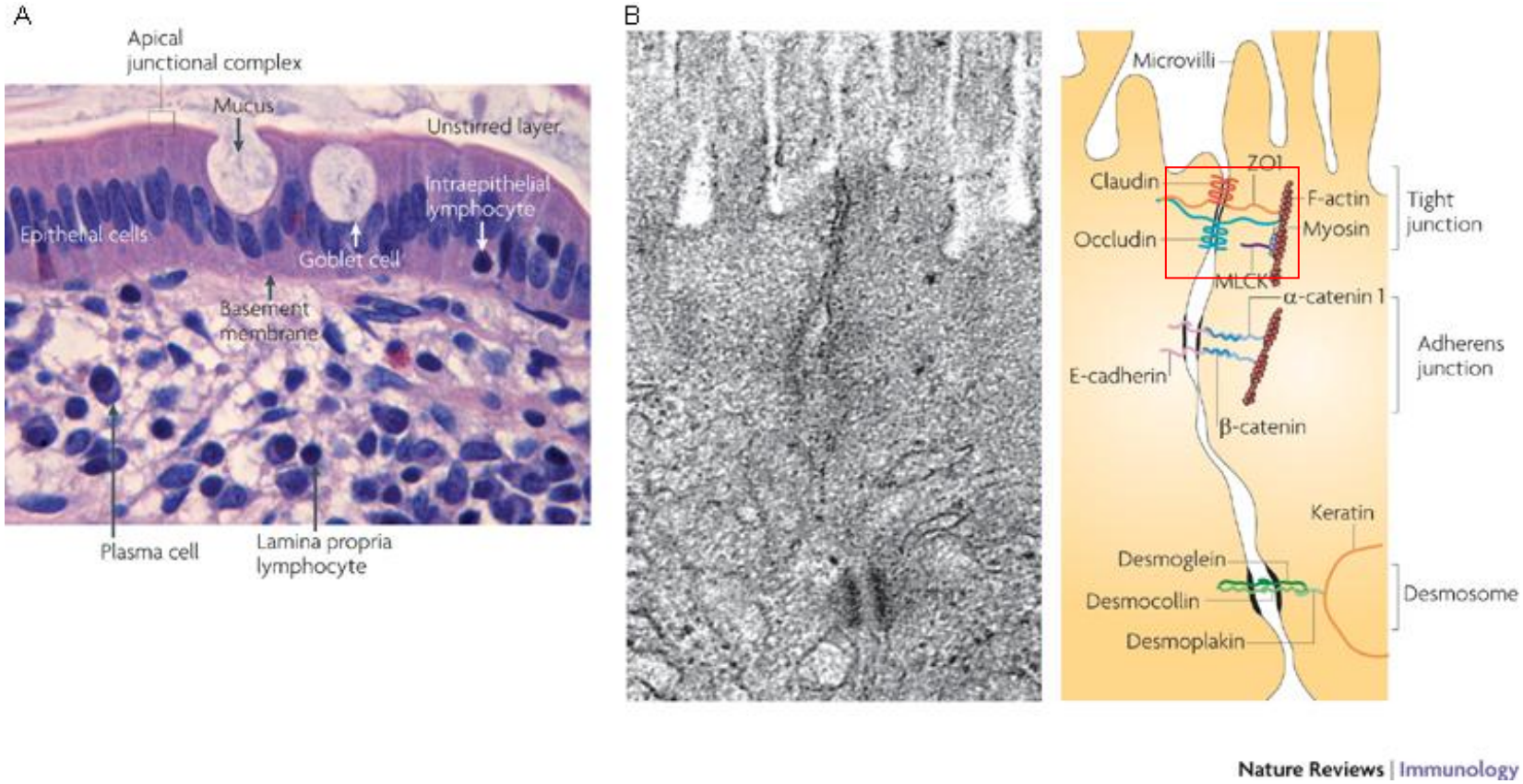
In addition, plasma proteins such as albumin, are inadvertently lost into the lumen in this way (Smith *et al.* 1987b; Yakoob *et al.* 1983) resulting in a significant reduction in albumin levels in draining lymph. As shown previously for pepsinogen (Smith *et*

*al.* 1983a; Smith *et al.* 1983b), the gastric lymph proteome would be expected to include proteins from the lumen and apical mucosal tissues of the abomasum, following passage across the mucosal barrier. In addition to the mast cell protease mentioned already (MMCP-1), it is quite possible that other proteins produced locally may alter this mucosal barrier, for example gelsolin, as discussed in the previous chapter. It is not known exactly what causes the "leak lesions" associated with this nematode infection, but a highly plausible explanation could be alteration in the tight junctions of the mucosal epithelium.

The mucosal barrier functions due to the presence of tight junctions (TJs). TJs, also called zonula occludens, form between two adjacent cells and in doing so, create a virtually impermeable barrier (Figure 6.1; (Turner 2009). Transmembrane proteins in adjacent cells can attach to one another directly, creating a branching network of strands which effectively form a seal between the cells. This seal forms at the junction between apical and basolateral regions of the plasma membrane in epithelial and endothelial cells, and takes the form of a continuous, circumferential belt-like structure. It is in this way that exchange of substances between the internal and external cellular environment is controlled (Forster 2008). In addition, TJs can control the movement of proteins within the plasma membrane, restricting their domain to either apical or basolateral surfaces (Rodriguez-Boulan & Nelson 1989) and hence creating specialised surfaces within what is otherwise a dynamic surface.

There are two groups of integral membrane proteins from which tight junction strands are created. These are the claudins and occludin (Figure 6.1).

**Figure 6.1: Adapted from Turner, 2009. Anatomy of the mucosal barrier. Figure A shows a histological section of human intestinal mucosa, with the most prominent features labelled. The area labelled Apical junctional complex is shown in more detail in Figure B (by electron micrograph and line drawing). The tight junction transmembrane proteins claudin and occludin can be seen anchoring to the underlying actin and myosin cytoskeleton through ZO-1 (red box). Also shown in Figure B are the adherens junction (maintain cellular proximity and intercellular communication) and desmosome (connect adjacent epithelial cells, points where keratin filaments attach to plasma membrane.)**

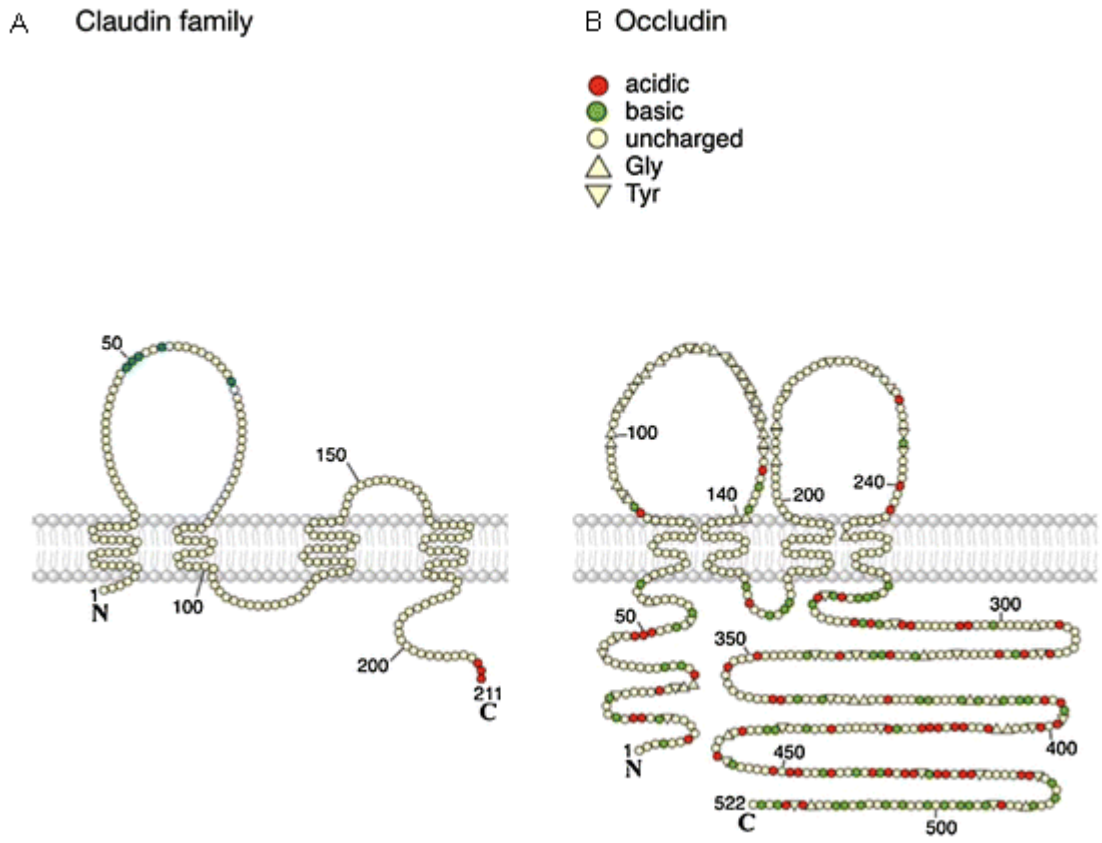


The claudins (Claudin 1 and 2) were first discovered in chicken liver extract by Furuse et al. (Furuse *et al*. 1998). To date, 24 claudins have been identified in various different species (Lal-Nag & Morin 2009). Claudin expression is tissue specific (Lal-Nag & Morin 2009; Morita *et al.* 1999), with most tissues expressing two or more claudins (Forster 2008). The claudins have a molecular weight of ~20-27 kDa, and are composed of four transmembrane domains (Tsukita & Furuse 2000). The N-terminus and C-terminus both sit in the cytoplasm and there are two external loops (Figure 6.2). The different claudin isoforms have variously charged external loops which interact to create pores which control the diffusion of ions through the paracellular space, with different claudins selectively allowing the passage of different charged ions (Furuse *et al.* 2001); (Tsukita *et al.* 2001). Due to the charge qualities created by the amino acid sequence of their extracellular loops, the combination of expressed claudin isoforms results in cell- or tissue-specific variations in the nature of barrier function (Tsukita *et al.* 2001).

The second main type of integral membrane protein involved in the formation of tight junctions is occludin. Occludin, like the claudins, was first identified in chicken liver extract (Furuse *et al*. 1993) and then later in humans and other mammals (Ando-Akatsuka *et al.* 1996). Occludin (~60kDa) also has four transmembrane domains (but shares no sequence similarity to the claudins), all of which sit within the N terminal half of the protein (Figure 6.2). However, whereas the charge dependent pore forming role of claudin is clear, the role of occludin in the tight junction complex remains undetermined (Forster 2008). Unlike the claudins, the extracellular loops of occludin are formed from an unusually high content of the amino acids tyrosine and glycine and are completely uncharged (Forster 2008). Various published studies have looked at the possible role of occludin in the tight junction. Over expression of occludin in mammalian cells in vitro results in reduced ion permeability (Balda *et al*. 1996) but increased permeability to mannitol, an uncharged compound (Balda *et al.* 1996). It has been suggested that occludin may be involved in regulating permeability (through its

interaction with claudin strands), with claudin strands forming the backbone of the tight junction strand (Forster 2008).

**Figure 6.2 : Image adapted from Forster, 2008. Transmembrane proteins of the tight junction. Figure A shows the claudin family. The claudins have a molecular weight of ~20-27 kDa, and are composed of 4 transmembrane domains (Tsukita & Furuse 2000). The N-termius and C-terminus both sit in the cytoplasm and there are 2 external loops Figure B shows murine occludin (the occludin amino acid sequence is highly conserved between species (Ando-Akatsuka *et al.* 1996)). The extracellular loops of occludin are formed with an unusually high content of the amino acids tyrosine and glycine (triangles) and are completely uncharged (Forster 2008).**



A further protein looked at in the abomasum for the purposes of this study is ZO-1. Z0-1 was first identified in a number of epithelial tissues, including colon, by Stevenson et al. (Stevenson *et al*. 1986) and belongs to a group of proteins called MAGUK (membrane associated guanylate kinase homologues) proteins. They identified a protein, of approximately 225kDa, which was localized to the tight junction area, occupying a peripheral membrane position (Stevenson *et al*. 1989) (unlike claudins and occludin, this is not a transmembrane protein). Due to its presence in all the tissues studied, Stevenson et al. concluded that ZO-1 is possibly a component of all tight junctions, in all types of epithelium and endothelium. ZO-1 has been shown to be closely associated with occludin and the underlying cytoskeleton ( F-actin) (Fanning *et al*. 1998; Furuse *et al*. 1994) (Figure 6.1), as well as another tight-junction specific protein, ZO-2 (Fanning *et al.* 1998). Fanning et al. (1998) proposed that the function of ZO-1 within tight junctions is organisational to link the other tight junction components to the actin cytoskelton.

There are thought to be two routes allowing passage across the tight junction. The first allows the passage of large particles, such as proteins and is termed the "leak pathway" (Turner 2009). Passage via the leak pathway shows no charge selectivity and is increased *in vitro* by the cytokines interferon-γ (IFNγ; (Watson *et al.* 2005) and tumour necrosis factor (TNF; (Wang *et al.* 2005). The alternative route is via small, charged pores (with charge being defined by the repertoire of claudin proteins(Turner 2009). Alterations in expression of the claudins therefore result in changes to the permeability of this pathway, and are influenced by external stimuli, such as cytokines (Turner 2009).

The aim of this chapter was to study the tight junction proteins occludin, claudin 1/3 and ZO-1 in the abomasal epithelium by means of immunofluorescence techniques. Abomasal tissues from infected sheep (both primary infection and immune challenged) were compared to uninfected, naïve abomasum. Observations were made on the distribution and relative abundance of these proteins to look for changes associated with

parasite infection which may account for the "leak-lesion" associated with *T.circumcincta* infection.

## *6.2 Results*

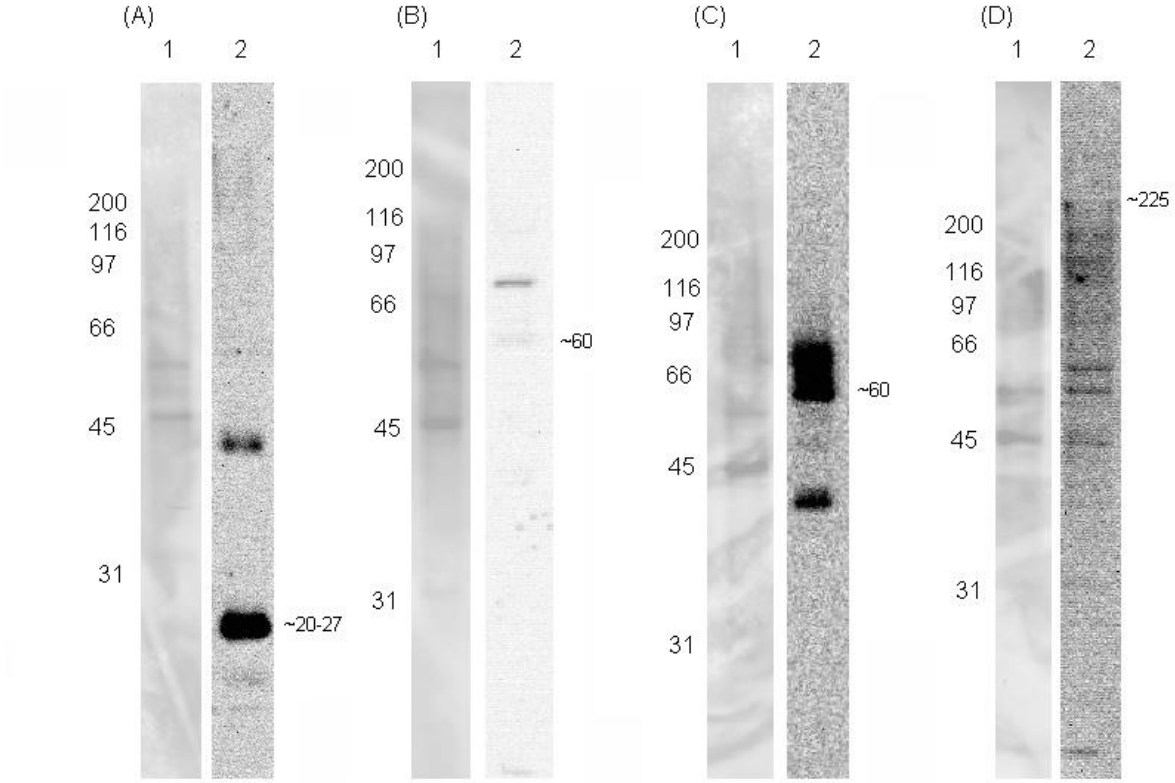
### 6.2.1 Verification of antibody sensitivity and specificity for ovine tight junction proteins

Using protein extracted from abomasal tissue from an uninfected sheep, the antibodies destined to be used in immunofluorescence were checked for specificity by western blot (Figure 6.3). This was necessary as these antibodies were not sheep specific and there was no published reference available where these antibodies had been used on ovine tissues. The antibodies used are shown in Table 2.1 (Materials and Methods).

Specific bands were evident on western blot at the expected MW for claudin 1/3 (Figure 6.3 (A)) and occludin-C (Figure 6.3(C)), showing both the specificity of the antibody, as well as the presence of these proteins in abomasal tissue. The western blot using occludin N terminal antibody gave only a faint band at the expected MW (~60kDa), with a more distinct band at a slightly higher MW of ~75kDa (Figure 6.3 (B)). This is likely to be due to hyperphosphorylation of the protein which has been shown before by western blot (Simonovic *et al.* 2000), and is also present when occludin-C terminal antibody is used, as shown by the extension of the band above the expected MW of the protein (Figure 6.3(C)). The western blot for ZO-1 antibody did not show a specific band at the expected MW. Possible reasons for this are discussed later.

**Figure 6.3: Coomasie stained 1-D SDS PAGE of abomasal protein extract (1) alongside western blot (2) using antibodies against the tight junction proteins claudin 1/3(A), occludin-N terminal(B), occludin-C terminal(C) and ZO-1(D). MW (kDa) deduced from markers ran on 1-D SDS PAGE alongside the abomasal protein extract (not shown) are indicated on LHS of each coomasie stained lane. The expected MW of each protein of interest is shown on the right side of the western blot lane in each instance.**

**Figure 6.3 Western blot of antibodies to be used for immunohistochemistry**



These antibodies were then used in immunofluorescence studies of abomasal sections (Materials and Methods section 2.12 and Table 2.2). Sections were studied from three groups of animals: naïve uninfected controls, day 5 post-infection of a primary infection, and day 5 post-infection in immune animals undergoing a challenge infection. Sections from six animals were examined for each group. For each section, five to ten areas of the epithelial surface were examined and one representative area was photographed and the image stored for later comparison.

The antibody specific to claudin 1/3 did not produce any signal in the abomasum. To ensure this was not a fault with either the antibody or the technique used, the same antibody and technique were used on a section of small intestine (ileum) and large intestine (rectum) from an uninfected sheep. Representative images obtained are shown in Figure 6.4. The antibody appeared to recognize the claudin protein in intestine and rectum, though the quantity of staining was relatively weak.

Both occludin antibodies (N and C terminal) were used and the sections examined. Both antibody probes give a similar staining pattern so only images stained with the anti-occludin N terminus antibody are shown. Figure 6.5 shows a representative image of abomasum, labelled with occludin-N terminal antibody, from each of the time points under investigation. On comparison of the six images from each group (control/Day 5 Primary/Day 5 immune), with those of each of the other groups, no difference in either the quantity of the protein, nor in the histological distribution of the protein could be determined.

Similarly, comparison of ZO-1 stained sections (Figure 6.6) did not show any notable change between the different infection groups compared to each other, or compared to the uninfected control.

**Figure 6.4: Histological sections from an uninfected sheep labelled with antibody specific for the tight junction protein claudin 1 and claudin 3 (antibody details in table 2.2, Materials and Methods) (x40 objective). Section A shows abomasal section, with the epithelial surface evident, but with no visible claudin labelling. Sections B and C show sections of ileum and rectum respectively. Claudin immunofluorescence (indicated by red fluorescence) is visible around the surface epithelial cells as evidenced by the red fluorescence. In all three sections, green labelling is due to sybr green and indicates cell nuclei.**

**Figure 6.4: Claudin 1/3**

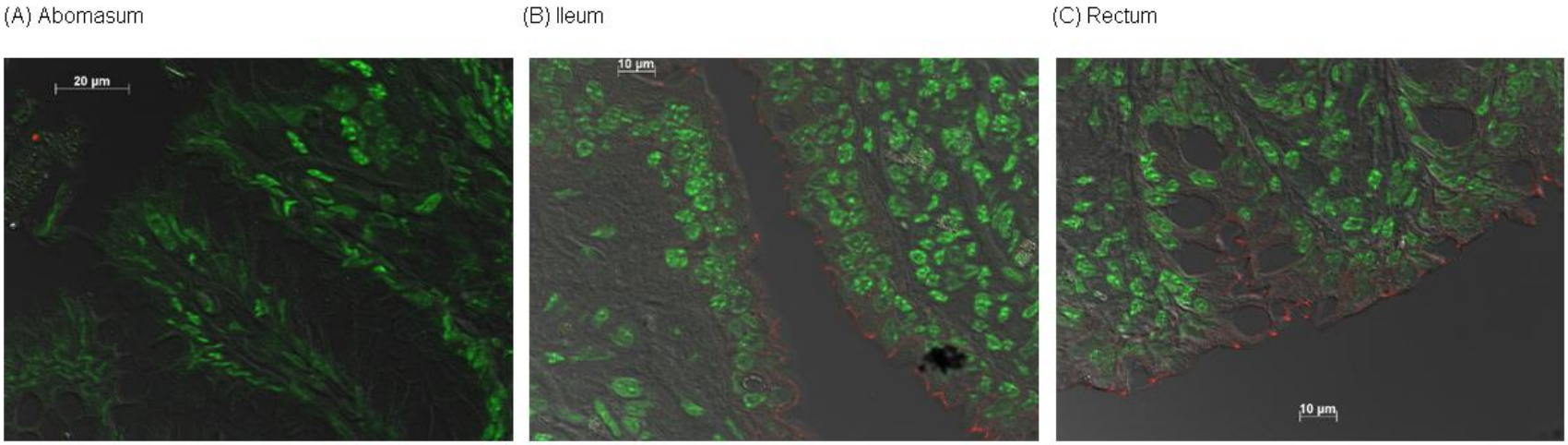


**Figure 6.5: Abomasal sections from uninfected naïve (A), day 5 post-infection (primary) (B) and day 5 post-infection (immune) (C) sheep, labelled with anti-occludin N-terminal antibody (x40 objective). Green labelling in all sections is due to sybr green detection of cell nuclei. The surface of the abomasal epithelium and gastric pits are evident in all sections. It is in these areas which come into contact with the lumen, that occludin labelling can be seen (red). Occludin labelling is largely confined to the apical surface of the epithelial cells, with some labelling in the luminal half of the lateral surfaces.**

**Figure 6.5: Occludin (N-terminal)**

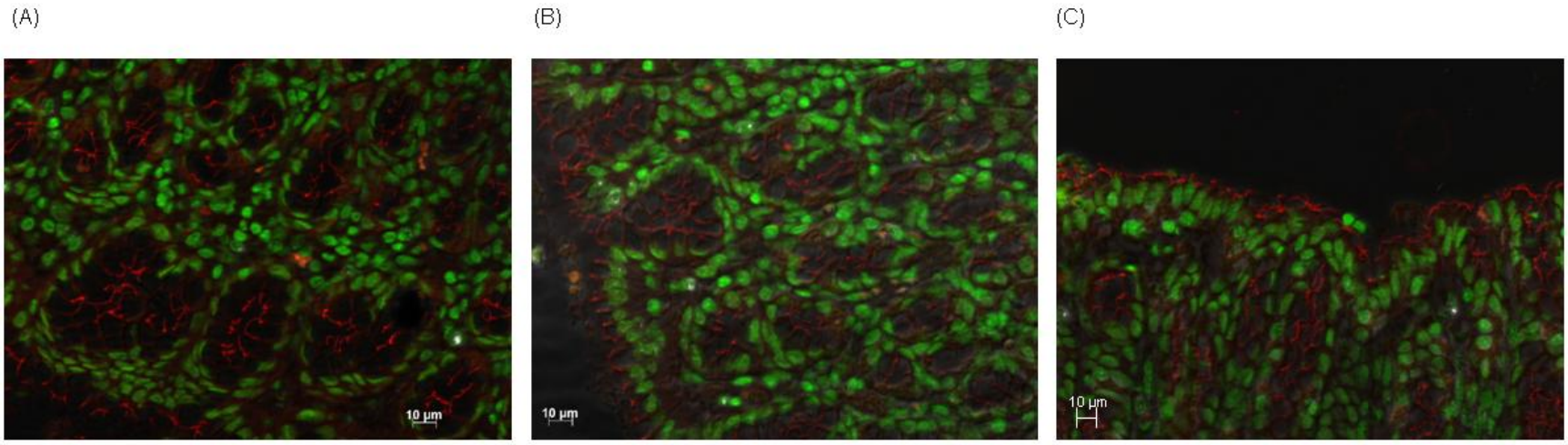
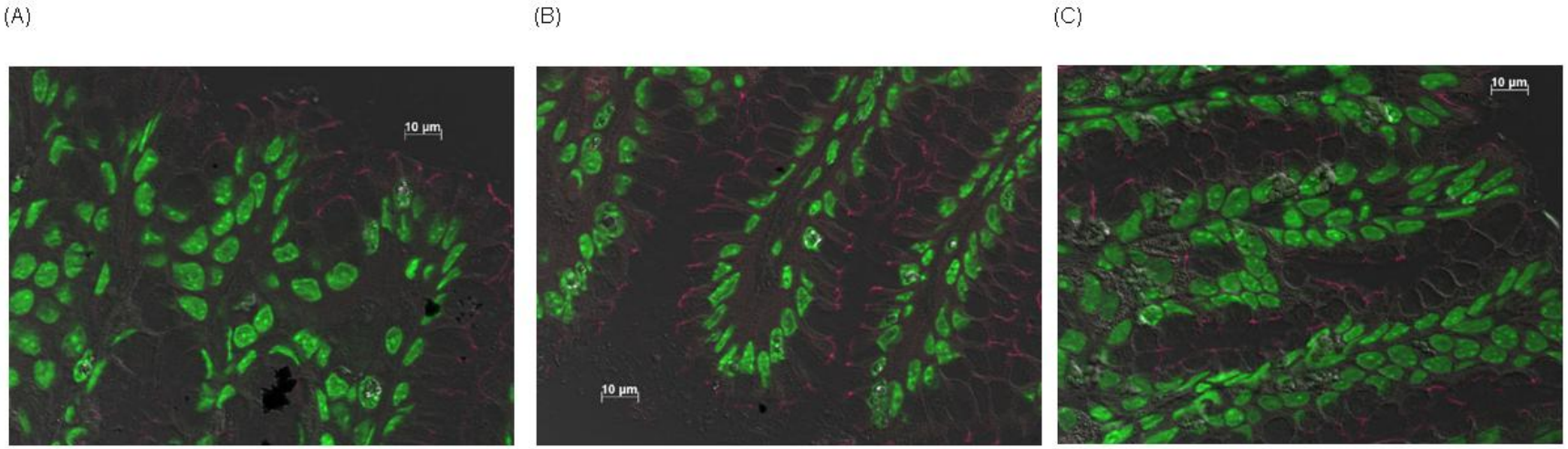


**Figure 6.6: Abomasal sections from uninfected naïve (A), day 5 post-infection (primary) (B) and day 5 post-infection (immune) (C) sheep, labelled with anti-ZO-1 antibody (x40 objective). Green staining in all sections is due to sybr green detection of cell nuclei. Abomasal epithelium is visible in all sections, with the ZO-1 protein (pink) being evident on the apical surface of the epithelial cells. Subtle differences in staining pattern and abundance between sections is evident, but this is not consistent when all sections in each group are compared.**

**Figure 6.6: ZO-1**



## 6.3 Discussion

Western blot analysis of abomasal protein extract was carried out to assess antibody specificity and sensitivity. These antibodies were then used for immunolocalisation studies of the tight junction proteins in the abomasa in different groups of sheep. The control group were naïve, uninfected animals. Day 5 primary infection animals are animals which were previously naïve, on day 5 post-challenge with *T.circumcincta* larvae. The immune challenged animals had been previously trickle infected, then that infection cleared prior to subsequent challenge with *T.circumcincta* (see Materials and Methods section 2.1). No discernible difference in either the quantity of the proteins or in the distribution of the proteins was found between groups.

Western blot analysis using the anti-claudin antibody showed a clear band at 20-27kDa (Figure 6.3A), the expected MW of the Claudin family. A second, fainter band was present at a MW of approximately 40-42kDa. It is likely that this is a dimer. The presence of dimers has been shown by immunoblots in other studies (Angelow & Yu 2009; Kojima *et al.* 2002). Occludin-N terminal antibody produced a faint band at 60kDa (Figure 6.3B), the expected MW of occludin. However, a more distinct band was present around 75kDa, possibly due to hyperphosphorylation. This has been shown by immunoblotting in other studies (Simonovic *et al*. 2000). The hyperphosphorylated form of occludin has been shown in intestinal epithelial cells to localise to the tight junction, whereas the non-phosphorylated form (lower MW band) represents occludin residing within the cell cytoplasm and/or basolateral membrane (Sakakibara *et al.* 1997; Wong 1997). Hyperphosphorylated forms of occludin were also indicated in the blot using occludin-C terminus antibody (Figure 6.3C). In addition, this blot also showed a band around 42kDa. It is possible that this is a result of proteolytic degradation. ZO-1 antibody produced multiple faint bands at varying molecular weights (Figure 6.3D), but failed to give a distinct band at the expected MW (~225kDa). It is possible that this antibody was not specific for ZO-1, or more likely (given the results from

immunohistochemistry), the protein did not run as expected on the gel. This protein is obviously of high MW and it may be that it was not effectively transferred to the PVDF membrane during the electroblotting step. Alternatively, the multiple faint bands evident may be due to proteolytic degradation of this protein. For all the antibodies used here, the proteins labelled could be confirmed by proteomic analysis of the peptides recognised by the antibodies.

The antibody specific to claudin 1 and 3 failed to show this protein in sections of uninfected abomasum (Figure 6.4 A). There was shown to be no fault with the antibody or technique due to the successful labelling of intestinal sections (Figure 6.4 B&C), showing the presence of this protein in the expected location. This result is unexpected given the presence of claudin in urea extracted protein from abomasal section (Figure 6.3 (A)). It is possible that a conformational feature of abomasal claudin prevented the antibody from binding when in a more native form in histological sections. Antigen retrieval using a different method may be beneficial in exposing the appropriate antigenic sites to the antibody.

The antibodies to occludin and ZO-1 stained the tight junctions of the abomasal epithelium. However, no consistent difference between groups was detected. This is in contrast to findings in mice infected with *Trichinella spiralis* where immunostaining revealed a change in occludin organization in uninfected versus infected mice (McDermott *et al.* 2003). McDermott et al. (2003) showed an obvious visual difference in occludin location, with a change from the expected plasma membrane location, to an intracytoplasmic location. This was accompanied by diminished levels of occludin (as shown by western blot) in infected mice (McDermott *et al.* 2003). These changes were not mirrored in ZO-1 or claudin-1 junctional proteins in *T.spiralis* infection (McDermott *et al.* 2003), but reduced expression of ZO-1 (and also occludin) was identified in *Strongyloides venezuelensis* infection of mice (Farid *et al*. 2008). These differences highlight the likelihood that different mechanisms are responsible for the increased

epthithelial permeability observed, even within a species, when the species of nematode varies.

The infected sample time point (day 5) was selected as this was the earliest possible time-point from available samples. However, it may be that tight junction pathology has not reached its maximum by day 5 in primary infection animals, (lymph pepsinogen maximum at day 12 post-infection, using a similar infection model (Smith *et al.* 1983b)). Alternatively, the presence of the "leak lesion" may have passed in the immune animals by day 5 (maximum lymph pepsinogen at day 2 post-infection (Smith *et al.* 1983a)). A sequential study looking at abomasum at multiple time-points from day 1 post-infection, until at least day 14 post-infection would help to solve the potential problem of "missing" the presence of the tight-junction lesion. This would obviously require great expense and the sacrifice of a large number of animals. Sequential biopsies may be a suitable alternative, but may in themselves produce pathology which is difficult to distinguish from that caused by the invading nematode (Scott *et al*. 2000).

The changes occurring in the tight junction proteins may be more subtle than can be observed by immunohistochemistry. Investigations in other infection models have focused on changes in phosphorylation pattern (Simonovic *et al.* 2000), on quantitative immunoblots (Zeissig *et al*. 2006), or on gene expression, by PCR (Farid *et al.* 2008; Kojima *et al.* 2002). Future study of the effect of *T.circumcincta* on tight junction proteins should perhaps involve a combination of all these techniques in order to determine whether it is indeed changes within the tight junction proteins that are responsible for the "leak lesion" phenomenon associated with *T.circumcincta* infection. In the absence of any evidence for gross alteration in tight junction protein distribution, an alternative mechanism, unrelated to tight junction proteins, cannot be ruled out at this stage.

# 7. Chapter 7: General Discussion

The parasitic nematode *Teladorsagia circumcincta* is the main cause of ovine parasitic gastroenteritis in temperate regions, including the UK. The clinical syndrome caused by infection with this nematode leads to poor productivity through reduced feed conversion efficiencies and slower weight gain. In the UK this is a major financial burden since the majority of the sheep farming industry is geared towards producing fat lambs for slaughter. Infection with *T.circumcincta* is also a welfare concern and so its control is essential if consumer confidence is to be maintained in farming standards.

Control of this parasite is principally through the use of anthelmintics at regular intervals throughout the year. Consumer concerns regarding drug residues, but more importantly, the emergence of anthelmintic resistant strains of parasite, is putting pressure on the industry to reduce reliance on these drugs. Anthelmintic resistance is a rapidly intensifying problem, with parasites showing resistance to all available anthelmintics ("triple resistant") becoming increasingly common (Bartley *et al.* 2004; Wrigley *et al.* 2006). Profitable sheep farming has become impossible on some farms due to the inability to control parasitic gastroenteritis. The development of new anthelmintics is a slow and costly process. One new class of drug (the amino-acetonitrile derivatives (AADs)) (Kaminsky *et al.* 2008b) has recently become available in some countries, but is not yet licensed in the UK (ZOLVIX®(Monepantel)-Novartis Animal Health). It is highly likely that the useful lifespan of this drug will also be limited by the emergence of resistant nematode populations.

Alternative methods of nematode control exist and are being developed, but may be better considered adjuncts to anthelmintic therapy, rather than stand alone solutions. These include the traditional method of grazing management, as well as the more recent research into biological control through the use of fungi (Larsen 2006), or genetic selection of nematode resistant animals (Windon 1996). None of these

alternative methods will give the same level of control as can be achieved by an effective anthelmintic drug. One possible solution which is hoped would give a similar degree of disease control is vaccination. Research into vaccination against *T.circumcincta* has been taking place for many years but to date progress towards an effective, commercially viable product has been slow (Knox & Redmond 2006). In part this is due to gaps in our knowledge concerning the immune responses responsible for eliminating the worms, and also how to induce such an appropriate response by means of vaccine delivered in a convenient form. A greater understanding of the mechanisms involved in natural immunity to *T.circumcincta* may aid in focussing the vaccine development to produce an appropriate, effective response.

It is thought that abomasal mucus is important in eliminating worms from immune sheep (Miller & Huntley 1982) and this process may also involve an immediate hypersensitivity type reaction, with mast cell degranulation (Knight *et al.* 2000; Miller & Huntley 1982). Antibodies are known to recognise parasitic antigens and are produced in response to infection (Bisset *et al.* 1996; Harrison *et al.* 2003a; Huntley *et al.* 2004), but their actual role as effectors is not clear. It is possible that IgA is involved in stunting of adult worms, and hence reduced fecundity (Halliday *et al.* 2007). Very little is known about the innate immune mechanisms acting within the mucosa.

This study aimed to investigate these innate mechanisms further, using gastric lymph as a means to investigate proteins present in the local environment of the abomasum. A broad overview of the proteins was taken by means of the proteomic analysis of lymph draining the abomasum, over the time-course of experimental infection with *T.circumcincta*. Lymph was studied from both immune animals facing a challenge infection, and naïve animals being challenged for the first time. This study served the purpose of highlighting proteins of potential interest which could be involved in either immunity or in pathology associated with infection. A number of these proteins were then investigated further by proteomic and molecular methods in order to establish their kinetics in response to infection, and so further define any potential

role they may have. An attempt was also made to look at the mucosal barrier as this is a crucial control point in determining the protein repertoire of the draining lymph and also of the local environment surrounding the worms.

In chapter 3 lymph draining from the abomasum was studied at three time points during experimental infection in both naïve sheep and immune animals undergoing challenge infection. The lymph was pre-fractionated prior to separation by 2-DE, by the removal of the most abundant proteins albumin and IgG. Immunodepletion of the most abundant proteins had been found to aid identification of the less abundant proteins when looking at a complex protein mixture, such as blood or lymph plasma (Omenn *et al*. 2005). Depletion was found to be efficient and specific, with very few non-target proteins being identified in the bound depletion column fractions, as identified by LC-ESI-MS/MS.

MALDI-TOF analysis of spots cut from 2-DE gels revealed the presence of many proteins not previously identified in lymph. The kinetics of all the visible protein spots were monitored over the time-course of experimental *T.circumcincta* infection. A consistent and statistically significant change in three proteins was found when all animals in the group were compared (n=5). These proteins were gelsolin (decreased significantly, $p<0.05$, day 21 cf. day 0 in primary infection), hemopexin (increased significantly, $p<0.05$, day 10 cf. day 0 in immune challenged) and α-1 B glycoprotein (decreased significantly, $p<0.05$, day 10 cf. day 0 in immune challenged). Following on from these results, gelsolin production within the abomasum was studied in more detail in chapter 5. As well as these statistically significant changes, alterations in the level of the acute phase proteins SAA and haptoglobin were also visible on the lymph gels from some animals. Because the levels of these proteins were not consistent between animals, these changes were not statistically significant, but interesting nonetheless. The acute phase proteins were investigated further in chapter 4.

Future proteomics work should include investigation of further depletion methods to allow greater loading of the less abundant proteins as it was found that loading was

limited by the likes of transferrin, meaning that proteins known to be present in lymph, for example intelectin (data not shown) were not visible. Exploring more recent technologies such as fluorescent labelling of samples prior to 2-DE may also advance the information to be gained from this type of broad approach. Fluorescent stains are more sensitive than conventional colloidal coomasie or silver staining, and the use of Difference Gel Electrophoresis (DIGE) excludes variation due to gel running artefacts (de Roos *et al.* 2008). Alternatively, a quantitative label-free mass spectrometry approach could be adopted (Wong & Cagney 2010), in combination with further sample pre-fractionation. Further investigation of α-1B glycoprotein, a lymph protein whose abundance was found to alter significantly during infection with *T.circumcincta*, may be of interest. This may be hampered by the lack of information and reagents to α-1B glycoprotein, but the production of a specific antibody to this protein may aid investigation, or local expression could be further investigated by PCR technologies.

Chapter 4 looked in more detail at the changes occurring in the acute phase proteins SAA, haptoglobin, and AGP. Acute phase protein levels were measured in lymph by specific assays. Levels of SAA showed a significant decline over the infection period, with haptoglobin showing a similar, but less distinct trend. The acute phase protein production is predominantly by the liver (Baumann & Gauldie 1994) but local production has been shown in other tissues (Eckersall *et al.* 2006; Lecchi *et al.* 2008). Due to potential confounding effects of surgery-related APPR, the local APPR was investigated by PCR. Preliminary PCR reactions were carried out to determine that these proteins were indeed transcribed locally. The PCR approach described here showed this to be the case, for the first time, in the abomasal mucosa. Further qPCR assays were carried out using abomasal tissue at various time-points following experimental infection of either immune or naïve sheep. SAA expression increased substantially at day 2 post infection in immune animals, compared to uninfected immune animals, and this trend continued to day 5. Haptoglobin expression was significantly higher at both day 2 and 5 post-infection in naïve challenged sheep compared to naïve uninfected, and significantly higher at day 2

immune challenged sheep compared to both uninfected naïve and uninfected immune. Local AGP expression was however unaltered by *T.circumcincta* infection.

The potential immunoregulatory functions that have been assigned to these proteins are wide ranging, and it is possible that SAA and haptoglobin expression changes are a result of the pathology created by the invading larvae. Alternatively, they may have a role in immunity to the invading larvae. Interestingly, haptoglobin is a protease inhibitor (Pagano *et al.* 1982; Snellman & Sylven 1967) and so may have a role in degrading parasitic enzymes, such as cathepsin F, which is the most abundant protein in *T.circumcincta* excretory/secretory products early in infection (Redmond *et al.* 2006; Smith *et al.* 2009). Upregulation of SAA expression may in turn increase expression of the mucin gene, MUC 3 (Larson *et al*. 2003) or recruit cells of the immune system to the site where they are required (Badolato *et al*. 2000).

The potential role of these proteins in *T.circumcincta* infection is varied and would make an interesting focus for future work. This could perhaps be investigated by the purification of these proteins and observations of effects in vitro directly on the nematode, on abomasal tissues and on the abomasal mucus. For example, the effect of haptoglobin on nematode invasion of the mucosa could be investigated using an in vitro organ challenge model (Athanasiadou *et al*. 2008). A similar model could be used to assess the effect of SAA on MUC gene expression.

Chapter 5 looked at local expression of gelsolin, one of the proteins found to change significantly in lymph from sheep infected with *T.circumcincta* over the experimental infection period. Gelsolin is a ubiquitously expressed, actin depolymerising protein, and as such has multiple roles (Bucki *et al*. 2008). It is a unique protein, being expressed in both a cytoplasmic and secreted form in humans, through differential splicing of the same gene (Kwiatkowski *et al.* 1988). Partial sequences for ovine gelsolin were obtained for three variants from abomasal tissues. A primer set common to all three was designed and used in a qPCR study to look at the effect of *T.circumcincta* infection on abomasal expression of this protein. Gelsolin expression was found to be significantly higher in immune uninfected sheep

compared to naïve uninfected sheep ($p<0.013$). Expression then fell rapidly such that day 2 post-infection immune animals showed significantly lower expression compared to day 2 uninfected immune animals. Higher gelsolin levels in immune sheep may allow for the faster release of secretory granules from goblet cells following challenge, compared to naïve sheep (Ehre *et al*. 2005). Once secreted, gelsolin protein could impact on mucus viscosity within the abomasum (Vasconcellos *et al.* 1994a) and so may have a role in worm expulsion (Miller & Huntley 1982).

Future work aimed at defining a role for gelsolin in *T.circumcincta* infection would be useful. Classification of transcripts into secreted and cytoplasmic forms would help to determine if the site of action is cytoplasmic or extracellular. Design of specific primers to distinguish between variants and qPCR assays on abomasal tissue could also be undertaken. Once it is established which variant of gelsolin is upregulated in the immune sheep, it will be easier to focus on potential roles. Defining the cell types most involved in gelsolin production would also be interesting with immunohistochemical staining of abomasal sections or, alternatively, using *in situ* hybridisation. Again, an *in vitro* organ challenge model may be of use for studying the function of this protein. If functional recombinant gelsolin was available its impact, if any, on goblet cell secretion could be studied.

Chapter 6 looked at the effect of *T.circumcincta* infection on the tight junction proteins ZO-1, Occludin and Claudin 1 and 3. Tight junctions create a barrier to free movement of macromolecules between cells and it is likely that disruption of these paracellular pathways leads to increased permeability of the mucosa as demonstrated by increased lymph pepsinogen levels (McKellar 1993; Simpson 2000). It is thought to be a mast cell mediated response through the actions of Th2 cytokines, particularly IL-4 (McDermott *et al*. 2003). Abomasal tissue sections from naïve uninfected controls and day 5 post-infection primary and immune challenged animals were examined by means of fluorescently labelled antibody. Staining was successful for ZO-1 and occludin, but failed using the anti-claudin 1 antibody, despite immunoblot analysis of abomasal protein extract suggesting this antibody had suitable specificity.

Examination of the tissue sections failed to show the relocation of occludin noted in *Trichinella spiralis* infection in mice ((Scudamore *et al*. 1998). No difference in either location or relative abundance of ZO-1 or occludin was observed from examination of immunofluorescently labelled abomasal tissue sections when compared to control slides. A more comprehensive time-course study may aid the study of the tight-junction protein response to *T.circumcincta* infection, but this would involve considerable animal sacrifice and at the time of carrying out these experiments, day 5 samples were the earliest time-point available.

Further investigation of these proteins still offers potential in defining the cause of the increased abomasal mucosal permeability observed in *T.circumcincta* infection. If time allowed, more comprehensive study of the abomasal tight junction proteins using multiple time points, and combining the methods of immunoblotting, immunofluorescence and PCR would be likely to yield further information as to the role of these proteins in immunity to or pathology caused by *T.circumcincta* infection. However, the possibility remains that leak lesions may not be tight junction related, which would be consistent with the lack of changes observed in these immunofluorescent studies.

The surgical procedure, and associated costs, required to obtain gastric lymph imposed limitations on the number of animals used for this work. This generally resulted in limited sample numbers which, in turn, reduced the power of the statistical analyses which could be applied. Despite this limitation, this study has highlighted proteins potentially involved in the host response to *T.circumcincta* infection. It has shown that there is a detectable local acute phase response to *T.circumcincta* infection. This is the first time such a response has been demonstrated to nematode infection. Gelsolin has also emerged as a protein worthy of further study given its known properties and that the work here indicates that expression is significantly higher in immune abomasa, compared to naïve tissue.

In summary, this study has taken a new approach to searching for potential important components involved in immunity to *T.circumcincta*. Although the cellular

components and targeted protein levels have been evaluated in gastric lymph from infected sheep in the past, such a broad overview of the protein repertoire of the tissue fluid bathing this organ, and the changes which occur in the levels of these proteins during infection, is a novel means of trying to identify potential immune effectors. This study has identified a number of proteins produced locally in the abomasum, worthy of further investigation as to their precise role in *T.circumcincta* infection.

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# A. Appendix: Statistics of 2DE gel analysis data

## *A1 Primary Infection*

**Table 1: Summary statistics of 2DE gel analysis data from animals undergoing a primary infection.**

All spots showing a 1.5 fold change or greater in more than one animal have been analysed statistically. Shown in red is the statistically significant result of interest.

| Protein | Day 0 (n=5) | | Day 11 (n=5) | | Day 21 (n=5) | | Analysis of Variance: P value |
|---|---|---|---|---|---|---|---|
| | Mean | SEM | Mean | SEM | Mean | SEM | |
| IgM | 2.70 | 1.10 | 2.681 | 0.221 | 2.485 | 0.857 | 0.964 |
| Albumin | 5.75 | 1.76 | 5.24 | 1.39 | 2.019 | 0.754 | 0.168 |
| α-1 antitrypsin | 9.56 | 1.08 | 8.82 | 1.57 | 12.47 | 1.79 | 0.236 |
| α-2 HS glycoprotein | 7.719 | 0.362 | 10.167 | 0.935 | 12.30 | 2.07 | 0.092 |
| Transferrin | 17.92 | 2.61 | 18.54 | 1.42 | 20.94 | 2.91 | 0.300 |
| Apolipoprotein A-1 | 5.62 | 1.29 | 5.37 | 1.43 | 6.37 | 1.37 | 0.748 |
| Hemopexin | 3.994 | 0.868 | 3.207 | 0.515 | 3.440 | 0.977 | 0.822 |
| Retinol binding protein | 0.5058 | 0.712 | 0.4324 | 0.0658 | 0.4488 | 0.0715 | 0.712 |
| SAA-(85) | 0.1392 | 0.0527 | 0.201 | 0.101 | 0.0526 | 0.0329 | 0.182 |
| SAA- (101) | 0.324 | 0.169 | 0.0992 | 0.0350 | 0.0648 | 0.0436 | 0.230 |
| α-1 B glycoprotein | 2.576 | 0.362 | 3.244 | 0.171 | 2.646 | 0.346 | 0.203 |
| Gelsolin | 0.707 | 0.111 | 0.3396 | 0.0815 | 0.3086 | 0.0947 | 0.042 |
| Apolipoprotein A-4 | 0.815 | 0.161 | 0.959 | 0.183 | 0.985 | 0.268 | 0.829 |
| Haptoglobin | 3.106 | 0.829 | 1.619 | 0.781 | 1.84 | 1.12 | 0.282 |
| Tetranectin | 0.0578 | 0.0171 | 0.0720 | 0.0259 | 0.0358 | 0.0210 | 0.176 |
| Ig lambda | 1.414 | 0.413 | 0.880 | 0.345 | 1.017 | 0.690 | 0.714 |
| Actin-2 | 0.2480 | 0.0615 | 0.1168 | 0.0561 | 0.1396 | 0.0639 | 0.324 |
| α-1 acid glycoprotein | 5.55 | 1.00 | 4.77 | 1.58 | 4.23 | 1.23 | 0.789 |
| IgJ chain | 0.3936 | 0.0434 | 0.546 | 0.132 | 0.3852 | 0.0923 | 0.234 |
| Zinc α-2 glycoprotein | 0.1326 | 0.0525 | 0.1820 | 0.0580 | 0.2178 | 0.0470 | 0.270 |

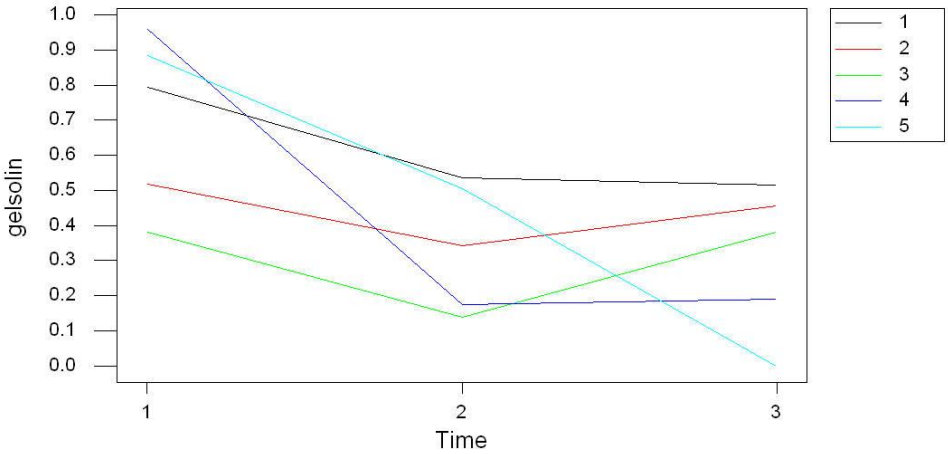
Data are spot volumes normalised to total spot volume (=100).

## *A2 Hypothesis testing: gelsolin*

### General Linear Model: gelsolin versus Sheep, Time

```
Factor     Type Levels Values
Sheep    random      5 1 2 3 4 5
Time      fixed      3 1 2 3

Analysis of Variance for gelsolin, using Adjusted SS for Tests

Source     DF     Seq SS     Adj SS     Adj MS       F      P
Sheep       4    0.14992    0.14992    0.03748    0.74  0.593
Time        2    0.49162    0.49162    0.24581    4.83  0.042
Error       8    0.40718    0.40718    0.05090
Total      14    1.04872

Tukey 95.0% Simultaneous Confidence Intervals
Response Variable gelsolin
All Pairwise Comparisons among Levels of Time

Time = 1 subtracted from:

Time     Lower    Center     Upper  ----+---------+---------+---------+--
2      -0.7752   -0.3676  0.040009    (-----------*-----------)
3      -0.8062   -0.3986  0.009009   (------------*----------)
                                    ----+---------+---------+---------+--
                                      -0.70     -0.35      0.00      0.35


Time = 2 subtracted from:

Time     Lower    Center     Upper  ----+---------+---------+---------+--
3      -0.4386  -0.03100    0.3766             (-----------*-----------)
                                    ----+---------+---------+---------+--
                                      -0.70     -0.35      0.00      0.35


Tukey Simultaneous Tests
Response Variable gelsolin
All Pairwise Comparisons among Levels of Time

Time = 1 subtracted from:

Level    Difference       SE of             Adjusted
Time       of Means  Difference   T-Value    P-Value
2           -0.3676      0.1427    -2.576     0.0756
3           -0.3986      0.1427    -2.794     0.0549

Time = 2 subtracted from:

Level    Difference       SE of             Adjusted
Time       of Means  Difference   T-Value    P-Value
3          -0.03100      0.1427   -0.2173     0.9744
```

Interaction plot for gelsolin:



## *A3 Immune challenged*

**Table 2: Summary statistics of 2DE gel analysis data from immune animals undergoing challenge infection.**

All spots showing a 1.5 fold change or greater in more than one animal have been analysed statistically. Shown in red are the statistically significant results of interest.

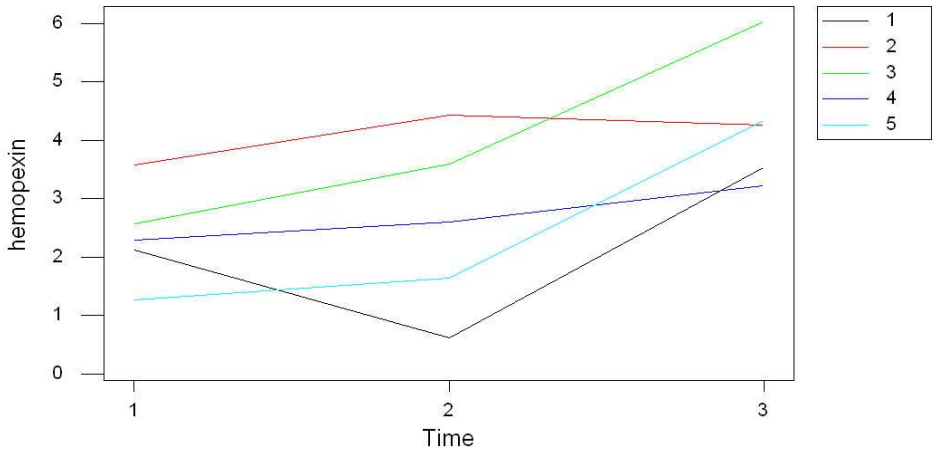
| Protein | Day 0 (n=5) | | Day 6 (n=5) | | Day 10 (n=5) | | Analysis of Variance: P value |
|---|---|---|---|---|---|---|---|
| | Mean | SEM | Mean | SEM | Mean | SEM | |
| IgM | 2.478 | 0.460 | 2.252 | 0.721 | 2.742 | 0.935 | 0.605 |
| Albumin | 9.16 | 1.47 | 4.11 | 2.12 | 4.02 | 1.12 | 0.014 |
| α-1 antitrypsin | 11.72 | 1.14 | 10.69 | 1.11 | 11.243 | 0.521 | 0.726 |
| α-2 HS glycoprotein | 11.431 | 0.875 | 11.98 | 1.52 | 12.514 | 0.922 | 0.328 |
| Transferrin | 26.28 | 2.73 | 22.16 | 3.24 | 25.37 | 2.96 | 0.412 |
| Apolipoprotein A-1 | 7.17 | 1.24 | 6.638 | 0.419 | 5.794 | 0.550 | 0.252 |
| Hemopexin | 2.370 | 0.373 | 2.579 | 0.678 | 4.277 | 0.484 | 0.016 |
| Retinol binding protein | 0.4288 | 0.0295 | 0.3770 | 0.0366 | 0.4076 | 0.0333 | 0.442 |
| SAA-(85) | 0.264 | 0.120 | 0.1580 | 0.0756 | 0.1400 | 0.0862 | 0.139 |
| SAA- (101) | 0.0416 | 0.0268 | 0.0226 | 0.0150 | 0.00860 | 0.00658 | 0.514 |
| α-1 B glycoprotein | 3.783 | 0.187 | 3.434 | 0.261 | 2.594 | 0.290 | 0.002 |
| Gelsolin | 0.265 | 0.136 | 0.139 | 0.127 | 0.1536 | 0.0988 | 0.671 |
| Apolipoprotein A-4 | 0.614 | 0.218 | 0.516 | 0.131 | 0.598 | 0.122 | 0.689 |
| Haptoglobin | 2.634 | 0.712 | 2.026 | 0.562 | 2.423 | 0.347 | 0.763 |
| Tetranectin | 0.0196 | 0.0113 | 0.0298 | 0.0218 | 0.0142 | 0.0132 | 0.697 |
| Ig lambda | 2.379 | 0.713 | 2.490 | 0.739 | 3.68 | 1.94 | 0.743 |
| Actin-2 | 0.1520 | 0.0897 | 0.0380 | 0.0171 | 0.0744 | 0.0358 | 0.206 |
| α-1 acid glycoprotein | 2.448 | 0.312 | 4.026 | 0.475 | 3.145 | 0.605 | 0.133 |
| IgJ chain | 0.3216 | 0.0616 | 0.419 | 0.100 | 0.3536 | 0.0716 | 0.737 |
| Zinc α-2 glycoprotein | 0.2218 | 0.0551 | 0.1314 | 0.0345 | 0.0724 | 0.0285 | 0.110 |

## A4 Hypothesis testing: hemopexin.

### General Linear Model: hemopexin versus Sheep, Time

```
Factor     Type Levels Values
Sheep    random      5 1 2 3 4 5
Time      fixed      3 1 2 3

Analysis of Variance for hemopexi, using Adjusted SS for Tests

Source     DF     Seq SS     Adj SS     Adj MS       F      P
Sheep       4    10.6382    10.6382     2.6595    3.53  0.061
Time        2    10.9364    10.9364     5.4682    7.26  0.016
Error       8     6.0222     6.0222     0.7528
Total      14    27.5968

Tukey 95.0% Simultaneous Confidence Intervals
Response Variable hemopexi
All Pairwise Comparisons among Levels of Time

Time = 1 subtracted from:

Time     Lower    Center     Upper  ----------+---------+---------+------
2       -1.359    0.2086     1.776   (---------*----------)
3        0.339    1.9066     3.474             (----------*---------)
                                    ----------+---------+---------+------
                                             0.0       1.5       3.0


Time = 2 subtracted from:

Time     Lower    Center     Upper  ----------+---------+---------+------
3       0.1304     1.698     3.266            (---------*----------)
                                    ----------+---------+---------+------
                                             0.0       1.5       3.0


Tukey Simultaneous Tests
Response Variable hemopexi
All Pairwise Comparisons among Levels of Time

Time = 1 subtracted from:

Level    Difference       SE of             Adjusted
Time       of Means  Difference   T-Value    P-Value
2            0.2086      0.5487    0.3801     0.9242
3            1.9066      0.5487    3.4745     0.0204

Time = 2 subtracted from:

Level    Difference       SE of             Adjusted
Time       of Means  Difference   T-Value    P-Value
3               1.698      0.5487     3.094     0.0353
```

Interaction plot for hemopexin:

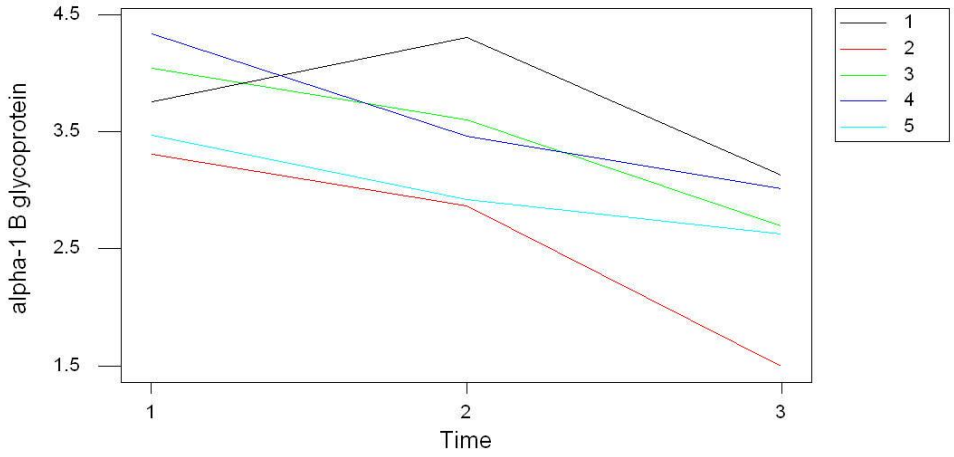


## A5 Hypothesis testing: α-1 B glycoprotein

### General Linear Model: alpha-1 B glycoprotein versus Sheep, Time

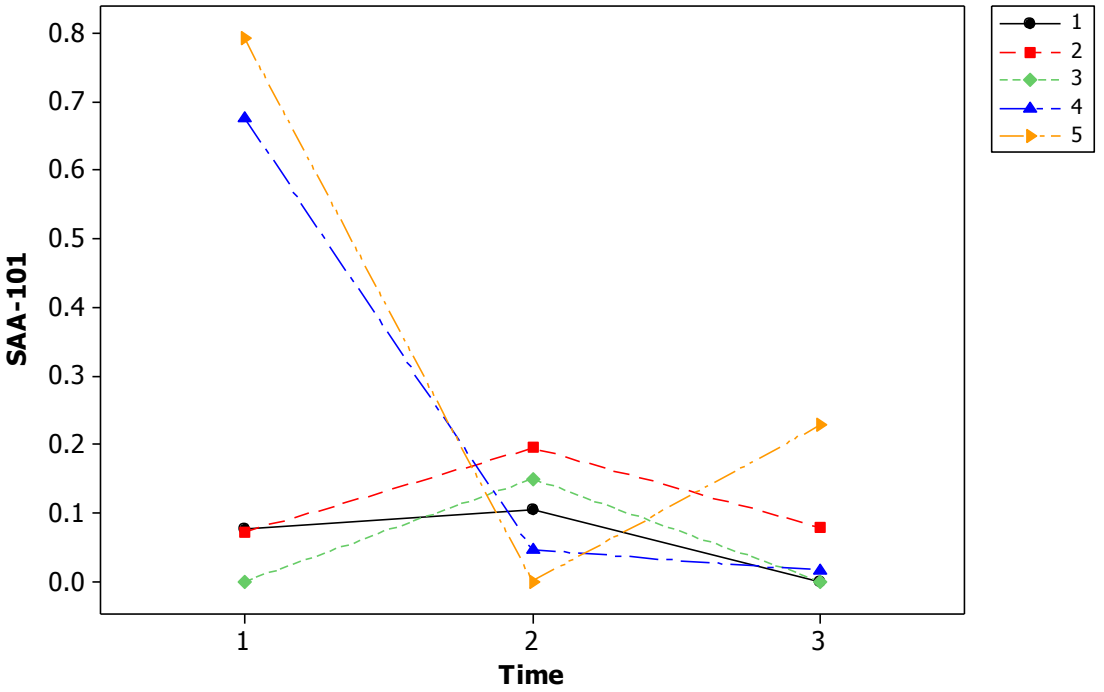
```
Factor     Type Levels Values
Sheep    random      5 1 2 3 4 5
Time      fixed      3 1 2 3

Analysis of Variance for alpha-1, using Adjusted SS for Tests

Source     DF     Seq SS     Adj SS     Adj MS       F      P
Sheep       4     2.8006     2.8006     0.7001    5.93  0.016
Time        2     3.7356     3.7356     1.8678   15.82  0.002
Error       8     0.9444     0.9444     0.1181
Total      14     7.4806

Tukey 95.0% Simultaneous Confidence Intervals
Response Variable alpha-1
All Pairwise Comparisons among Levels of Time

Time = 1 subtracted from:

Time     Lower    Center     Upper  -+---------+---------+---------+-----
2       -0.970    -0.350    0.2712                (---------*----------)
3       -1.810    -1.189   -0.5684   (---------*----------)
                                    -+---------+---------+---------+-----
                                  -1.80     -1.20     -0.60      0.00


Time = 2 subtracted from:

Time     Lower    Center     Upper  -+---------+---------+---------+-----
3       -1.460   -0.8396   -0.2188         (---------*---------)
                                    -+---------+---------+---------+-----
                                  -1.80     -1.20     -0.60      0.00


Tukey Simultaneous Tests
Response Variable alpha-1
All Pairwise Comparisons among Levels of Time

Time = 1 subtracted from:

Level    Difference       SE of             Adjusted
Time       of Means  Difference   T-Value    P-Value
2            -0.350      0.2173    -1.609     0.2966
3            -1.189      0.2173    -5.473     0.0015

Time = 2 subtracted from:

Level    Difference       SE of             Adjusted
Time       of Means  Difference   T-Value    P-Value
3          -0.8396      0.2173    -3.864     0.0118
```

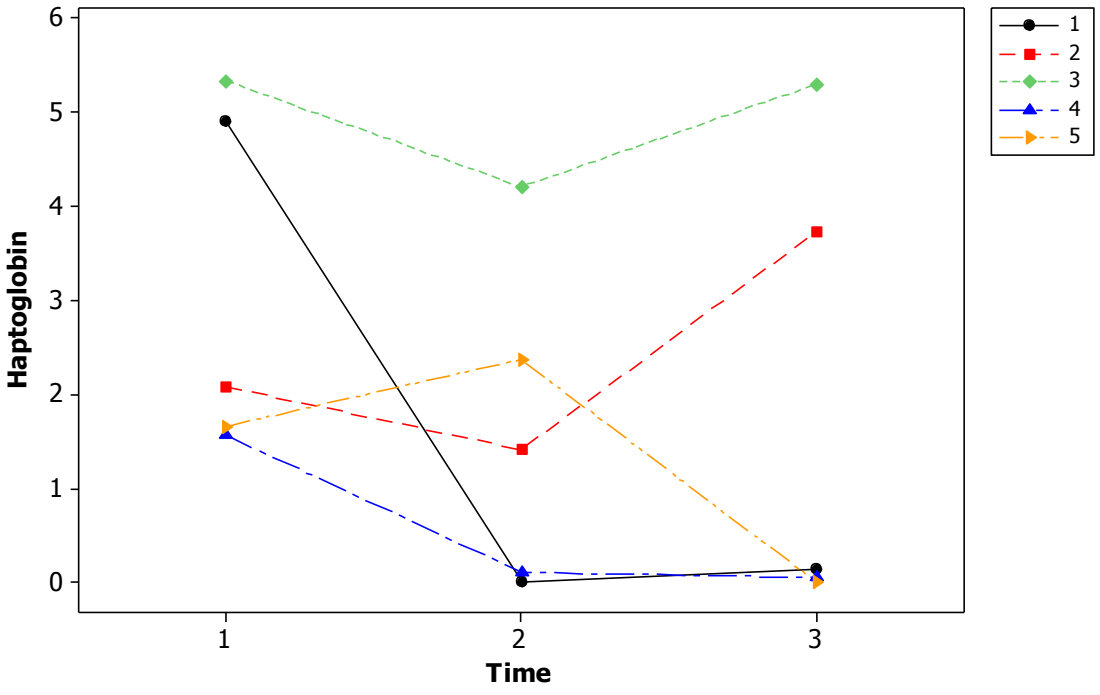
Interaction plot for α-1 B glycoprotein:



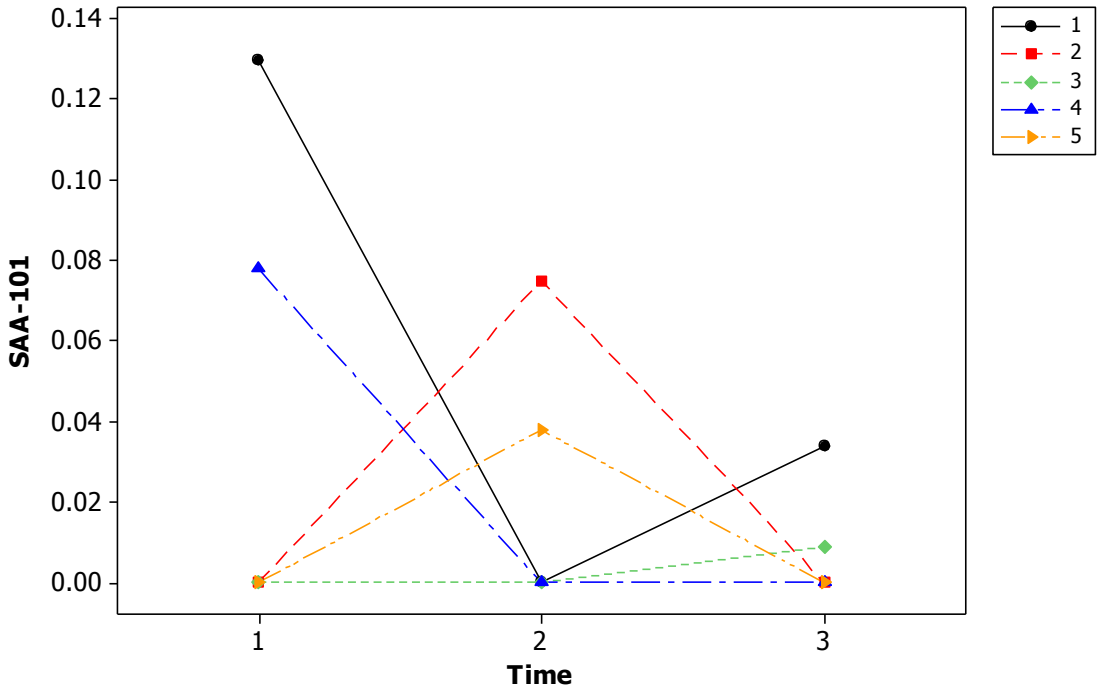
## A6 Acute phase proteins : Interactions plots

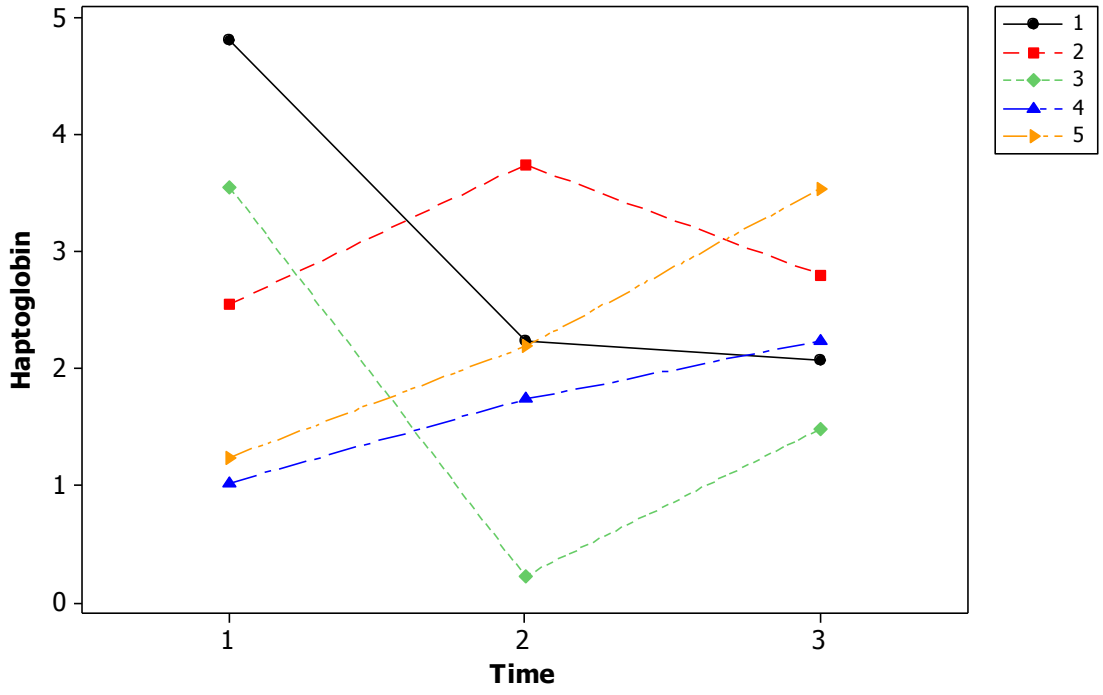
Primary Infection



Immune challenged



# Appendix 2: Publication